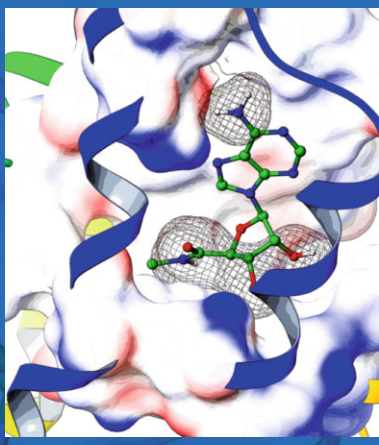
Pier Andrea Borea
Editor



A_3 Adenosine Receptors

from Cell Biology to Pharmacology and Therapeutics

Springer

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Cover illustration: "MECA" (5′-N-methylcarboxamidoadenosine) docked to the human A_3 receptor. Cover image was kindly provided by Dr. Andrei Ivanov, National Institutes of Health, Bethesda, MD

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Preface

This book, with its 16 chapters, documents the present state of knowledge of the adenosine A_3 receptor. It covers a wide range of information, including data from studies of theoretical, molecular and cellular pharmacology, signal transduction, integrative physiology, new drug discoveries and clinical applications. It fills an important gap in the literature since no alternative source of such information is currently available. Although the A_3 receptor is increasingly being recognized for its increasing number of biological roles throughout the body and many A_3 receptor ligands have proven useful in elucidating peripheral and central pathologies, many issues remain unresolved. Moreover, research activity in this field continues to grow exponentially, resulting in a constant flow of new information. The chapters in this book cover both basic science and the relevant applications and provide an authoritative account of the current status of the field. They have enabled my goal as editor to make "A_3 Adenosine Receptors from Cell Biology to Pharmacology and Therapeutics" an up to date, scientifically excellent, reference source, attractive to basic and clinical scientists alike, a reality.

Detailed understanding of the physico-chemical aspects and molecular biology of the A_3 receptor provides a solid basis for its future development as a target for adenosine-based pharmacotherapies (Chapters 2 and 3). Recognition and characterization of intracellular pathways modulated by A_3 receptor activation supports the belief that modulating these signaling routes is likely to lead to considerable advances in the management of many diseases (Chapters 4 and 5). The identification of new potent and selective A_3 receptor ligands opens new frontiers for the elucidation of the therapeutic potential arising from stimulating or blocking the A_3 receptor (Chapters 1, 6, 7 and 8). The A_3 receptor appears to play a prominent role under ischemic conditions and remains a promising target for promoting angiogenesis and treating neurodegenerative diseases associated with acute ischemia (Chapter 9). In terms of clinical utility, it will be critical to explore in greater detail the efficacy of the A_3 receptor-mediated protective response in diseased hearts, particularly with respect to diabetes, hypertension, hypertrophy, and dyslipidemias (Chapters 10 and 14). The important protective role of the adenosine A_3 receptor, originally discovered in studies of ischemia-reperfusion injury in the heart, has now been extended to skeletal muscle (Chapter 13). The importance of eosinophils in allergy and asthma is well recognized and targeting the A_3 receptor for the

treatment of eosinophil-dependent pulmonary diseases such as asthma, chronic obstructive pulmonary disease and rhinitis offers realistic hope of novel therapies (Chapters 1 and 11). A_3 receptors are present in many immune cells and are involved in the regulation of inflammatory and immune processes, suggesting new therapeutic strategies may emerge for inflammatory conditions such as sepsis, asthma and autoimmune disorders including rheumatoid arthritis, Crohn's disease and psoriasis (Chapter 12). The oral bioavailability of certain A_3 agonists and encouraging data from early clinical studies support the development of these agents as anti-rheumatic drugs (Chapter 15). The effectiveness of the A_3 receptor agonist, Cl-IB-MECA, in several animal tumor models led to the introduction of this molecule into a program of pre-clinical and clinical studies. The excellent safety profile led to the initiation of clinical studies in patients with hepatocellular carcinoma which are currently ongoing. Paradoxically, it appears that A_3 receptor antagonists can also be considered promising in the treatment of human cancer (Chapter 16). These exciting results arising from the hard work of the people associated with this book hold promise for a future generation of new drugs for the treatment of important diseases.

I would like to express my gratitude to the distinguished contributors who have expressed their confidence in this book by contributing to it and who will be key players in the success of the research on A_3 receptors in the future. 2011–2012 will mark the 20th anniversary of the cloning of the A_3 receptor. It would give me enormous pleasure if new molecules targeting the A_3 receptor could become drugs at this time with the help and participation of the eminent scientists who have authored this book.

I would also like to thank very sincerely the Publishing Editor of Springer Biomedicine, Dr. Max Haring. It has been a pleasure working with him in this project. I am also extremely grateful for the critical contributions by Dr. John Fozard who has played a key role in the entire project. Last, but certainly not least, I wish to thank the members of my Research Group for their scientific work in the field of A_3 adenosine receptors.

The first edition of "A_3 Adenosine Receptors from Cell Biology to Pharmacology and Therapeutics" volume is dedicated to my wife Cristina and to all the friends and colleagues who contributed to this book.

Ferrara, Italy Prof. Pier Andrea Borea

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Part I
Introduction

Chapter 1
From Hypertension (+) to Asthma: Interactions with the Adenosine A_3 Receptor from a Personal Perspective

John R. Fozard

1.1 Introduction

There have been several detailed accounts of the structure, biological functions and ligands of the A_3 receptor in the recent literature (Fredholm et al. 2001a; Jacobson and Gao 2006; Press et al. 2007; Gessi et al. 2008; Hasko et al. 2008) and the contents of this book will certainly add significantly to bringing our knowledge of this site up to date. There seems little point, therefore, in following tradition in an introductory chapter such as this and writing a 'Past, Present and Future of the field' type of article. I have, therefore, decided to risk the charge of self-promotion and give an account of my own personal 'interaction' with the A_3 receptor. This started before the A_3 receptor was discovered as a offshoot of an interest in adenosine A_1 receptor agonists as a novel approach to the treatment of hypertension and emerged, chameleon-like, almost a decade later as part of a concept for the treatment of allergic asthma. Those of my readers who have in the past, or are currently, working to define the biological relevance of the A_3 receptor will surely recognise in what follows some of the unique challenges posed by this intriguing site.

1.2 Homage to the Discoverers of the A_3 Receptor

The A_3 receptor was identified during the G-protein receptor cloning frenzy of the early 1990s. Although I had no part in its discovery, in recognition of those who set the scientific ball rolling for so many of us, I summarise the two seminal papers in which the discovery was described. In 1991, Meyerhof and colleagues reported the isolation of a cDNA clone encoding a novel putative G-protein coupled receptor

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from a rat testis cDNA library. A corresponding 1.5 kb mRNA was expressed exclusively in the testis localized in spermatocytes and spermatids but not in spermatogonia, Leydig or Sertoli cells. Although the ligand for this receptor was not identified, the authors, understandably, speculated that the receptor, designated tgpcr1, may have a role in male reproduction (Meyerhof et al. 1991). In 1992, Zhou and colleagues described several cDNA sequences from rat striatum that encoded G-protein coupled receptors, one of which, designated R226, was identical to tgpcr1 (Zhou et al. 1992). On the basis of the sequence homology in its transmembrane domains with the adenosine A_1 (58%) and A_{2A} (57%) receptors and its capacity to bind adenosine receptor agonist ligands, Zhou and colleagues concluded that R226 encoded an adenosine receptor which they designated the A_3 receptor. They confirmed the high expression of the receptor in the testis but, importantly, also showed low-level mRNAs to be present in the lung, kidney, heart and parts of the central nervous system implying that the A_3 receptor could have more widespread biological significance than simply to modulate testicular function. It seems remarkable that the biological significance of the A_3 receptor present in the testis has never been established. Despite the fact that biochemical readouts show a number of cell types in the rat testis to respond functionally to A_3 receptor stimulation (Rivkees 1994), mice lacking the A_3 receptor breed with no difficulty (Salvatore et al. 2000). Despite this reassuring finding the presence and significance of the A_3 receptor in human testis remains unknown and it cannot be assumed that a selective A_3 receptor ligand put forward for clinical development would be free of effects on male reproductive function.

1.3 Hypertension (+)

1.3.1 A Cardiovascular Response to Adenosine Receptor Ligands in the Rat That Is Not Mediated by A_1 or A_2 Receptors

In the late 1980s, whilst at the Preclinical Research Department of Sandoz in Basel, I had an interest in adenosine and its receptors based on the belief that selective A_1 receptor agonists could be exploited as novel antihypertensive drugs. The project, known as hypertension (+), was aimed at identifying compounds which not only lowered the elevated blood pressure but had beneficial effects on other aspects of the condition. In this context, A_1 receptor agonists were considered attractive since such agents would be expected to lower blood pressure without causing reflex tachycardia, to suppress plasma renin, to reduce plasma free fatty acid and triglyceride concentrations and to increase insulin sensitivity, all of which could bring significant benefits in the treatment of hypertension. A highly selective A_1 receptor agonist, SDZ WAG-994, arose from this work which was used to confirm the concept both in preclinical studies (Wagner et al. 1995) and in early clinical development. For pharmacokinetic reasons, however, SDZ WAG-994 did not progress in development.

At the outset of our A_1 receptor project, the fall in blood pressure induced by A_1 receptor agonists in the rat was assumed to be primarily a consequence of the intense bradycardia associated with the hypotensive response (see Webb et al. 1990 and references therein). As part of the support studies during the development of SDZ WAG-994, we felt it important to explore in further detail the mechanism of the blood pressure fall resulting from A_1 receptor activation. To this end, we analysed the cardiovascular effects in the rat of N^6-cyclopentyladenosine (CPA), a commercially available, reasonably selective, A_1 receptor agonist. In some experiments, in order to eliminate reflex cardiovascular effects and thus simplify the interpretation of the data, we used the pithed preparation with blood pressure raised to normal with an infusion of angiotensin II. As expected, in such preparations, intravenously administered CPA powerfully reduced heart rate and there was an associated fall in blood pressure. To our surprise, however, whilst the bradycardia could be blocked by the broad spectrum adenosine receptor antagonist, 8-(p-sulphophenyl)theophylline (8-SPT) the blood pressure fall induced by CPA was resistant to blockade with a maximal dose of this agent. Two obvious conclusions followed from this: First, the bradycardia could not be the explanation for the fall in blood pressure and second, as we concluded at the time, the blood pressure fall was 'unlikely to be mediated by A_1 or A_2 receptors' (Fozard and Carruthers 1993a).

In late 1992, the paper of Zhou and colleagues describing the discovery and biological properties of the rat A_3 receptor appeared (Zhou et al. 1992). Their description of a new adenosine receptor at which alkylxanthine-type adenosine receptor antagonists were at best weakly active, provided an obvious possible explanation for the 8-SPT-resistant fall in blood pressure induced by CPA. A key finding of Zhou et al. was that that the A_3 receptor could be labeled with high affinity by the agonist radioligand, I^{125}APNEA (N^6-2-(4-amino-3-iodophenyl)ethyladenosine). In our pithed rat preparation, we found that low doses of the non-iodinated derivative, APNEA, induced hypotensive responses which were unaffected by high doses of 8-SPT. Similar responses were seen with NECA (5′-N-ethylcarboxamidoadenosine) and the **R** and **S** enantiomers of PIA (N^6-phenylisopropyladenosine) (Fozard and Carruthers 1993b). Xanthine insensitivity, high potencies of APNEA, NECA and **R**-PIA and an enantiomeric selectivity favouring **R**- over **S**-PIA were the distinguishing features of the A_3 receptor described by Zhou et al. (1992). Further analysis disclosed that the 8-SPT-resistant fall in blood pressure induced by APNEA was suppressed by pertussis toxin (Carruthers and Fozard 1993a), which implicates inhibitory Gi/Go G-proteins in the response as is the case for the coupling mechanism of the cloned receptor. The response was also blocked, by BW-A522 (3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine (Fozard and Hannon 1994), which exceptionally for a xanthine derivative, shows nM affinity and high selectivity for the sheep and human equivalent of the rat A_3 receptor (Linden et al. 1993). Thus, we felt confident in concluding that activation of the A_3 receptor initiates a fall in blood pressure in the rat. However, we did not know the target cell(s) involved in the response.

1.3.2 The Hypotensive Response to A_3 Receptor Ligands in the Rat Is Mast Cell Dependent

Although we were able to show that a fall in systemic vascular resistance and a decrease in cardiac output was the basis of the 8-SPT-resistant fall in blood pressure induced in the rat by APNEA (Salzmann and Fozard 1994), we did not know whether APNEA acted directly on elements of the cardiovascular system and/or indirectly by modulating transmitter or mediator release. Possibly favouring the latter option was the latency of onset of action of APNEA of several seconds (Fozard and Carruthers 1993a) which was not seen with a variety of other vasodepressor agents in this preparation (Carruthers and Fozard 1993b) and would be entirely consistent with a delay due to activation of an intermediary mechanism. Moreover, a plausible candidate for such a role had recently been identified; cells of the cultured rat mast cell line, RBL-2H3, contained the A_3 receptor activation of which facilitated the release of allergic mediators induced by allergen (Ramkumar et al. 1993). We therefore set out to test the hypothesis that the fall in blood pressure induced by A_3 receptor activation in the rat involved the mast cell. The key results from a comprehensive analysis (Hannon et al. 1995, 2002a; Fozard et al. 1996) are summarised below. They provided convincing evidence that mast cells throughout the body are the target cell involved in adenosine A_3-receptor mediated hypotension in the rat.

- Hypotension induced by APNEA could be mimicked by the mast cell degranulating agent, compound 48/80.
- Neither APNEA nor compound 48/80 induced cardiovascular effects in animals depleted of their mast cell mediators by repeated dosing with compound 48/80.
- Hypotension induced by APNEA could be blocked by the mast cell stabilizing agents, disodium cromoglycate and lodoxamide.
- Plasma and serum histamine concentrations were markedly increased associated with the hypotensive effects of APNEA.
- APNEA induced rapid and widespread mast cell degranulation in (e.g.) connective tissue, thymus, mesenteric lymph node, kidney, skin and diaphragm.

1.3.3 Comments on the Significance of Adenosine A_3 Receptor-Induced, Mast Cell Degranulation In Vivo

Naturally, the above data had repercussions for our selective A_1 receptor agonist approach to the treatment of hypertension. When 8-SPT-resistant hypotension was used as an indicator of A_3 receptor activation, the selectivity of the available, nominally selective A_1 receptor agonists was substantially less with respect to the A_3 receptor than the A_{2A} or A_{2B} receptors (Carruthers and Fozard 1993b; Fozard and Carruthers 1993a, b). Although at the time it was not known whether the human mast cell responded to A_3 receptor stimulation with degranulation, it was recognized that a highly selective A_1 receptor agonist may be needed to avoid a potentially

dangerous activation and degranulation of mast cells. SDZ WAG 994 was therefore designed to be sufficiently selective that complications arising from A_3 receptor activation would have been unlikely (Wagner et al. 1995).

Second, our data had (and retain) significance for the interpretation of results obtained with A_3 receptor adenosine receptor agonists in in vivo studies in rodents and possibly other species. For example, studies with the selective A_3 receptor agonist, IB-MECA (N^6-(3-iodobenzyl)adenosine)-5′-*N*-methyl carboxamide), implicate the A_3 receptor in behavioural depression in mice (Jacobson et al. 1993) and both post ischaemic brain damage (Von Lubitz et al. 1994) and seizure susceptibility (Von Lubitz et al. 1995) in gerbils. However, scratching in mice which could be blocked by the 5-hydroxytryptamine/histamine receptor antagonist, cyproheptadine, and long lasting hypotension in gerbils suggests that extensive mast cell degranulation is occurring under the conditions of these experiments. More recently, cytokine modulation induced by 2-Cl-IB-MECA (2-chloro-((N^6-(3-iodobenzyl) adenosine)-5′-*N*-methyl carboxamide) in mice treated with endotoxin has been shown to be mediated by histamine released from mast cells (Smith et al. 2002). Histamine was also released by 2-CI-IB-MECA in studies on myocardial ischaemia/reperfusion injury in mice although this was not the basis of the cardioprotection (Ge et al. 2006). In general, however, the effects observed in rodents with A_3 receptor agonists are likely to reflect the polypharmacology of A_3 receptor activation plus the effects of the mediators released from mast cells. Importantly, such data would be of limited relevance, if any, to the human where the A_3 receptor appears to play no role in mast cell degranulation (Gessi et al. 2008; Hasko et al. 2008; Wilson 2008).

Finally, we showed that NECA (a non-selective adenosine receptor agonist), CGS 21680 (2-[p-2-(carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine – a selective A_{2A} adenosine receptor agonist) and several nominally selective A_1 receptor agonists induced hypotensive responses in the pithed rat in the presence of a high dose of 8-SPT which fully blocks the A_1 receptor mediated bradycardia (Carruthers and Fozard 1993b; Fozard and Carruthers (1993a, b). These data indicate that significant activity at the (rat) A_3 receptor is a widespread property amongst adenosine receptor ligands traditionally used to discriminate between adenosine A_1 and A_2 receptor subtypes and suggest prudence in the use of these agents as pharmacological tools.

1.4 Antagonists of the A_3 Receptor for the Treatment of Asthma

1.4.1 Background and Concept

In 1994, I took over the leadership of the asthma group in preclinical research in Sandoz, Basel. Perhaps because of our previous focus on the link between adenosine

A_3 receptor activation and mast cell degranulation, I was intrigued by the fact that the airways of allergic asthmatics were much more sensitive to inhaled adenosine (or more usually adenosine monophosphate – AMP, used for convenience because of its superior solubility) than the airways of non-asthmatics and that the resulting bronchoconstriction appeared to be mast cell mediated (for a comprehensive and balanced review see Holgate 2005). As mentioned above, the facilitation of mast cell mediator release induced by allergen by activation of A_3 receptors had been demonstrated in rat RBL-2H3 cells (Ali et al. 1990; Ramkumar et al. 1993). In in-house experiments using guinea-pig lung or in vivo in the guinea pig we identified a remarkable potentiation of the bronchoconstrictor response to allergen by activation of A_3 receptors (Fig. 1.1).

Together these findings spawned a concept, 'The response of the airway mast cells to allergen is determined by adenosine acting through A_3 receptors' and, in 1995, a programme, 'Antagonists of the A_3 receptor for the treatment of asthma', was initiated based on the following considerations:

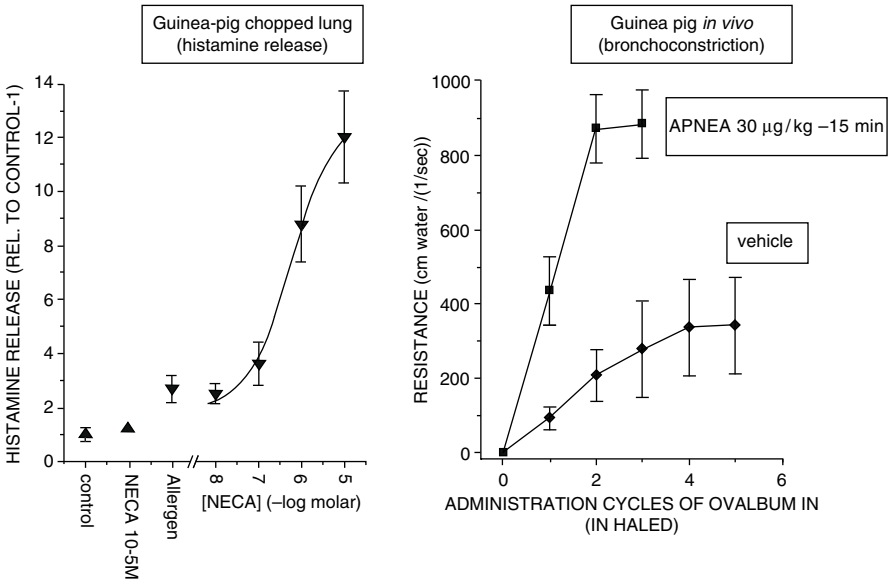

Fig. 1.1 Interaction between allergen and adenosine receptor agonists with respect to histamine release from guinea-pig chopped lung (*left*) or bronchoconstriction in the guinea pig in vivo (*right*). Tissues and animals were passively sensitized to ovalbumin. In the histamine release assay, a threshold response to allergen is augmented concentration-dependently by NECA. In the whole animal, a single intravenous injection of APNEA markedly enhances the bronchoconstrictor response to allergen (J.R. Fozard and H.J. Pfannkuche, unpublished observations 1994)

- Adenosine has a pivotal role in the pathophysiology of asthma.
- Adenosine facilitates preformed mediator and possibly cytokine release from mast cells by activating A_3 receptors.
- Antagonists at the A_3 receptor would prevent periodic exacerbations of asthma and lead over the longer term to a reduction in airways inflammation and bronchial hyperresponsiveness.

With this brief, our chemistry colleagues set out to design antagonists with potency and selectivity at the A_3 receptor and we biologists went off to devise mechanistic and/or disease models in which their molecules could be evaluated.

1.4.2 The Design and Synthesis of Novel Potent and Selective A_3 Receptor Antagonists

To rapidly identify a compound class with potential affinity at the human A_3 adenosine receptor, a diverse library of compounds was obtained and high throughput screening initiated. N-[4-(4-methoxyphenyl)-thiazol-2-yl]-acetamide came out as a hit and structure-activity relationship studies led rapidly to the synthesis of a number of aminothiazole derivatives, exemplified by N-[5-pyridin-4-yl-4-(3,4,5-methoxyphenyl)-thiazol-2-yl]-acetamide, with subnanomolar antagonist activity at the human A_3 receptor and greater than 1,000-fold selectivity over the other adenosine receptor subtypes (For full details see Press et al. 2004). Moreover, this compound was a selective antagonist of the hypotensive response to the prototype A_3 receptor agonist, 2-Cl-IB-MECA, in the rat indicating that blockade of the A_3 receptor could be obtained in vivo.

1.4.3 An Example of the Species Selectivity of the A_3 Receptor: The Receptor Responsible for Adenosine Augmentation of Mediator Release from Human Mast Cells Is Not the A_3 Receptor

At about the time that the efforts of the chemists to synthesise selective A_3 antagonists had begun to bear fruit (1995/1996), the assumption implicit in our concept that human mast cells would behave like those of the rodent was called into serious question. The first (and key) observations came from Feoktistov and Biaggioni (1995) who provided evidence that in the human mast cell line, HMC-1 (which although derived from a patient with mast cell leukaemia shows some biochemical characteristics similar to the mast cells of the lung (Feoktistov et al. 1998)), the A_{2B} receptor and not the A_3 receptor is responsible for the potentiation of phorbol

12-myristate 13-acetate (PMA)-induced augmentation of IL-8 release. With hindsight, there were already at least two observations in the literature which did not accord with a facilitatory role for the A_3 receptor in human mast cells. First, Hughes et al. (1984) showed that responses of mechanically dispersed human lung mast cells to adenosine following immunological challenge with anti-IgE had the features of an A_2 receptor with respect to agonist relative potencies and blockade by low concentrations of 8-phenyltheophylline. Second, Peachell et al. (1991) showed that adenosine and its analogues potentiated mediator release induced by anti-IgE from passively sensitised human lung mast cells and here too the response was susceptible to blockade by 8-phenyltheophylline.

In May 1997, based on an increasing awareness of the importance of the A_{2B} receptor on human mast cells (see Feoktistov and Biaggioni 1997a), we decided to refocus our programme and set potent and selective blockade of the human A_{2B} receptor as the major criterion for identifying a compound for possible clinical development. The A_3 receptor was retained as a target for several reasons (based on the information available at the time). First, A_3 receptors had been shown to be present on human eosinophils and to couple to signalling pathways that lead to cell activation (Kohno et al. 1996; observations subsequently supported by Reeves et al. 2000). Since asthmatic inflammation is characterised by extensive infiltration of the airways by activated eosinophils, it is possible that the elevated adenosine concentrations associated with asthma would contribute to eosinophil activation through stimulation of A_3 receptors. Second, activation of A_3 receptors mediates inhibition of eosinophil chemotaxis (Knight et al. 1997). Since adenosine levels are highest at the site of inflammation, A_3 receptor activation could be pro-inflammatory by inhibiting eosinophil migration away from the sites of inflammation. Last, but not least, the chemists had built up considerable expertise in designing A_3 receptor antagonists. It was decided that either a dual A_{2B}/A_3 antagonist or a selective A_{2B} antagonist would be considered relevant for clinical evaluation.

1.4.4 The Design of Mixed A_{2B}/A_3 Receptor Antagonists and Their Biological Evaluation In Vitro

As described above, a series of 5-pyridylaminothiazoles had been designed and synthesised as highly potent and selective antagonists at the adenosine A_3 receptor (Press et al. 2004). It was essentially an extension of the structure-activity relationship to involve 5-imidazo and 5-triazolo substituted aminothiazoles which enabled the rapid identification of several dual A_{2B}/A_3 receptor antagonists with acceptable selectivity over the A_1 and A_{2A} receptors. Of the lead compounds, the mesylate salt of 3-[5-(methylimidazol-1-yl)-2-(pyrazin-2-ylamino)-thiazol-4-yl benzonitrile (QAF805) was considered to be the superior compound (Compound 5f – Press et al. 2005).

QAF805 has high affinity for the human recombinant adenosine A_{2B} receptors and shows selectivity for these sites over the human A_1 and A_{2A} receptors (55- and 522-fold,

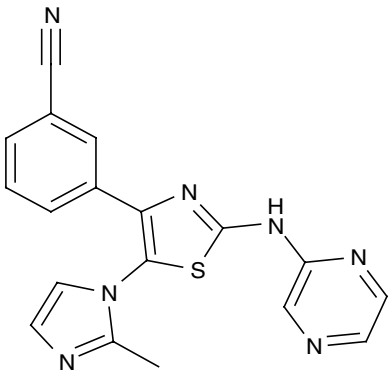

Table 1.1 Affinities of QAF805 for human adenosine receptor subtypes

A_1[a]	A_{2A}[b]	A_{2B}[c]	A_3[d]
K_i (nM)	K_i (nM)	K_B (nM)	K_i (nM)
186 ± 38 (5)	1775 ± 548 (5)	3.4 ± 0.2 (3)	10.2 ± 0.4 (5)

Values represent means ± s.e. mean of the number of experiments indicated in parentheses.[a] Radioligand binding assay with ^{3}H-DPCPX.[b] Radioligand binding assay with ^{3}H-ZM 241385.[c] Reporter gene assay.[d] Scintillation proximity assay with ^{125}I-AB-MECA (Press et al. 2005).

respectively). QAF805 also shows high affinity for the human A_3 receptor and is 18- and 174-fold selective for this site relative to the human A_1 and A_{2A} receptors, respectively (Press et al. 2005; Table 1.1). In functional models of the rat, dog and guinea pig A_{2B} receptors (Fozard et al. 2003a), QAF805 was a silent, surmountable antagonist yielding K_B values close to the Ki values from the human receptor binding assay (8 ± 1; n = 5), 1 ± 0.2 (4) and 7 ± 1 (4) nM, respectively). It bears emphasis that QAF805 was somewhat more potent as an antagonist of the rat A_1 receptor mediating contraction of the rat spleen (Fozard and Milavec-Krizman 1993) (K_B 42 ± 7 nM, n = 4) than at the human A_1 receptor (Ki from radioligand binding assay 186 nM – Table 1.1). QAF805 was inactive in a broad screen against other receptor and enzyme targets and had a good in vivo pharmacokinetic profile when given orally in the rat (Press et al. 2005).

1.4.5 A Second Example of the Species Selectivity of the A_3 Receptor: The In Vivo Evaluation of QAF805

Whilst the in vitro evaluation of QAF805 had been relatively straightforward, the in vivo evaluation was more of a challenge since at that time (1999) there were no disease-relevant animal models available to detect antagonist activity at human or indeed rodent A_{2B} or A_3 receptors. Moreover, there was no in vitro assay available

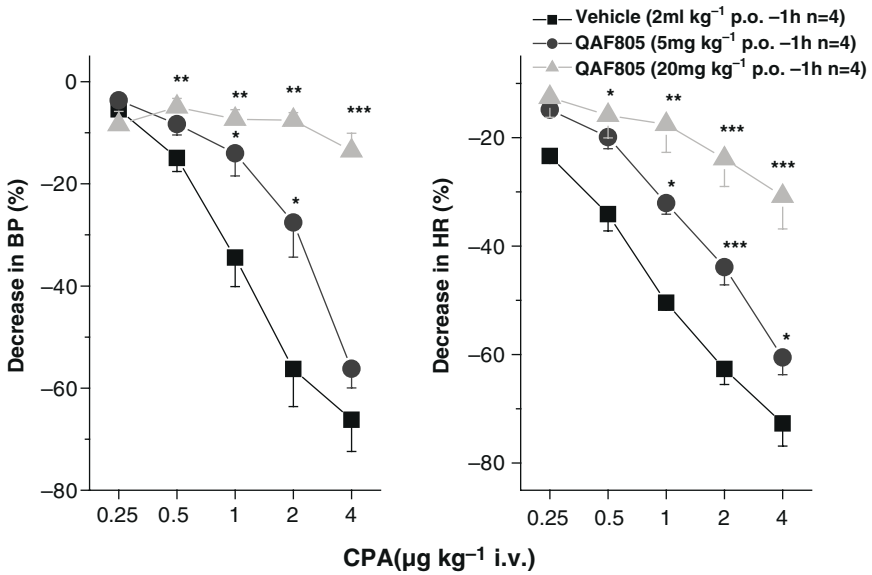

Fig. 1.2 The effect of QAF805 on the cardiovascular response to CPA in the rat. Shown are the decreases in blood pressure (BP) and heart rate (HR) induced by CPA (0.25, 0.5, 1, 2, and 4 μg kg^{-1}; cumulative doses i.v.). Vehicle or NVP-QAF805 was given orally 1 h prior to CPA. Results are expressed as means ± s.e.mean. **p < 0.01, ***p < 0.001; indicates significant difference by comparison with vehicle-treated animals (J.R. Fozard and L. Mazzoni, unpublished observations 1999)

to us to measure activity at the rat A_3 receptor. Thus, our initial strategy was to confirm that we could show A_3 receptor blockade in vivo and for this we returned to our rat model where activation of A_3 receptors results in mast cell-mediated hypotensive responses. The result was convincing although disappointing. At an oral dose of 10 mg kg^{-1}, QAF805 completely blocked the cardiovascular response to the selective A_1 receptor agonist, CPA (Fig. 1.2), but had no effect on the fall in blood pressure induced by the selective A_3 agonist, 2-Cl-IB-MECA (Fig. 1.3). Thus, unlike the A_{2B} receptor which manifests no species selectivity with respect to the antagonist potency of QAF805, QAF805 shows high affinity for the human A_3 receptor (Table 1.1) but is at best a very weak antagonist at the rat A_3 receptor.

1.5 Modelling the Airways Response to Adenosine: An Atypical Receptor Mechanism Mediates the Bronchoconstrictor Response to Adenosine Augmented Following Allergen Challenge

During the time that QAF805 was being identified and profiled as a potential candidate for clinical development, we had been working to design a disease model which would be predictive for clinical activity. Our conceptual starting point was

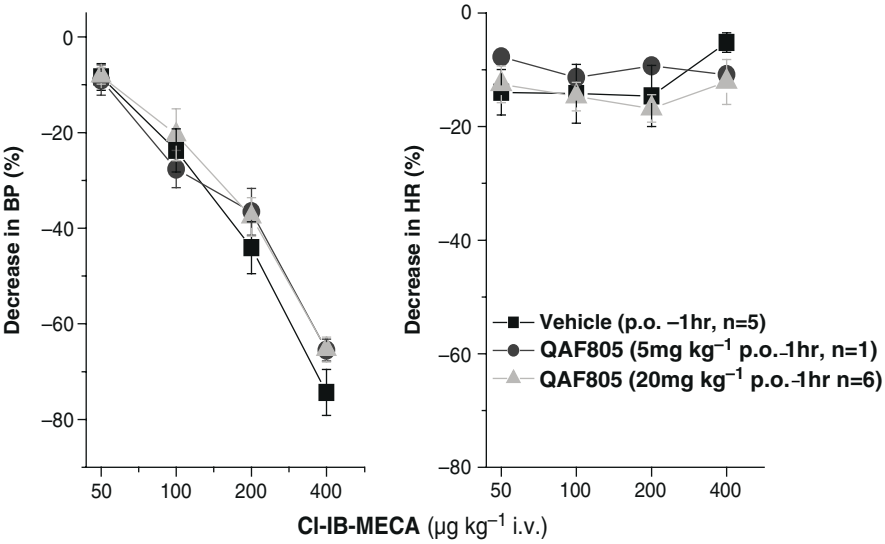

Fig. 1.3 The effect of QAF805 on the cardiovascular response to 2-Cl-IB-MECA in the rat. Shown are the blood pressure (BP) and heart rate (HR) changes induced by 2-Cl-IB-MECA (50, 100, 200 and 400 µg kg^{-1}; cumulative i.v.). Vehicle or QAF805 was given orally 1 h prior to Cl-IB-MECA. Results are expressed as means ± s.e. mean from n individual animals (J.R. Fozard and L. Mazzoni, unpublished observations 1999)

the striking difference between asthmatic and non-asthmatics with respect to their sensitivity to adenosine and the fact that the majority of asthmatics are allergic. Certainly, there was ample evidence from animal studies, including our own data from the guinea pig (Fig. 1.1), of the synergy between adenosine receptor activation and allergen in inducing mast cell degranulation, although the receptor subtype involved varies with the species (Marquardt et al. 1984; Ramkumar et al. 1993; Auchampach et al. 1997; Fozard and Hannon 2000; Salvatore et al. 2000). Thus, we decided to explore whether the response to adenosine augmented following allergen challenge could be used to model the airways response to adenosine in asthmatics. We chose to work with the Brown Norway rat since this strain readily and consistently forms IgE following active sensitisation, exhibits both early and late responses, and develops pulmonary inflammation and bronchial hyperreactivity following exposure to allergen (Elwood et al. 1991; 1992; 1993; Renzi et al. 1993, 1996). To accurately mimic the clinical situation the bronchoconstrictor response to adenosine was required to manifest the following:

- Upregulation associated with allergic pulmonary inflammation
- Mast cell dependency
- Tachyphylaxis
- Selective (partial) blockade by theophylline
- Acute blockade by glucocorticosteroids
- Evidence for A_{2B} and/or A_3 receptor mediation

In a comprehensive analysis we found that all the criteria were met with the exception of the receptor subtype(s) involved in the response (Hannon et al. 2001, 2002a, b; Fozard et al. 2003b).

Since rat mast cells respond to adenosine through the A_3 receptor (Ali et al. 1990; Ramkumar et al. 1993), we fully expected this to be reflected in our pharmacological analysis of the mast cell-dependent, bronchoconstrictor response to adenosine. However, when agonist potencies were compared only the non-selective agonist, NECA, was able to mimic the effect of adenosine. CPA (A_1 selective), CGS 21680 (A_{2A} selective) or 2-Cl-IB-MECA (A_3 selective) neither separately nor in combination mimicked adenosine despite evidence from heart rate and/or blood pressure changes that the respective receptors for which these agents show selectivity were being stimulated (Fig. 1.4; Hannon et al. 2002b).

Thus, surprisingly, the data did not support a role for the A_3 receptor. Even more surprisingly they appeared to implicate the A_{2B} receptor in the response! The plot thickened when further pharmacological analysis using antagonists failed to support the involvement of the A_{2B} receptor. I give two examples from a comprehensive analysis (Hannon et al. 2002b). First, 8-SPT and CGS 15943 (9-chloro-2-(2-furanyl[1,2,4] triazolo[1,5-c]quinazolin-5-amine) are antagonists at A_1, A_{2A}, and A_{2B} but not A_3

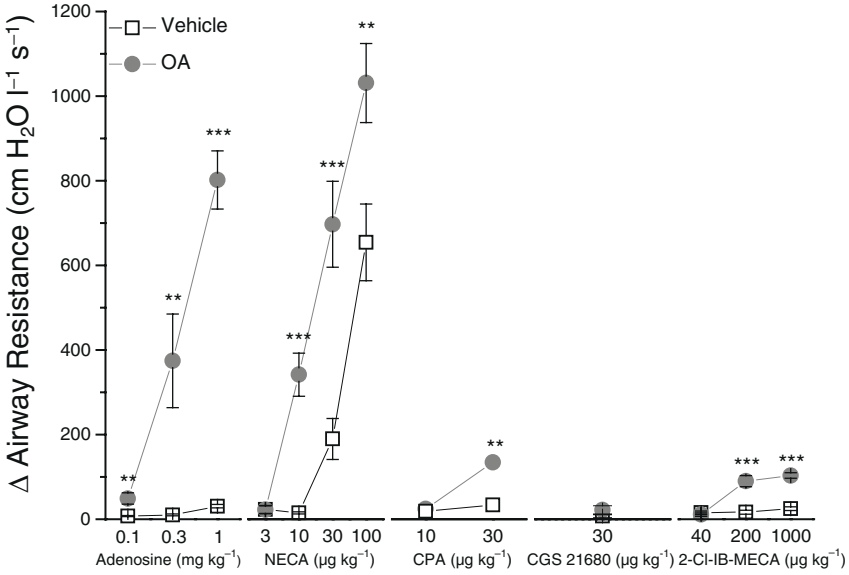

Fig. 1.4 The bronchoconstrictor effects of adenosine, NECA, CPA, CGS 21680 and 2-Cl-IB-MECA in actively sensitised, Brown Norway rats 3 h post intratracheal instillation of vehicle (saline, 0.2 ml) or ovalbumin (OA, 0.3 mg kg^{-1}). The agonists were given i.v. and to avoid tachyphylaxis, only one response was generated per animal. Results are expressed as means ± s.e. means (n = 4–5). $^{**}P < 0.01$, $^{***}P < 0.001$ that the value is significantly different from the equivalent value in the vehicle-challenged group (from Hannon et al. 2002b, with permission)

receptors. Although both compounds inhibited the bronchoconstrictor response to adenosine, the degree of blockade (approximately threefold) did not reflect the plasma concentrations, which were 139 and 21 times greater than the K_B value at the rat A_{2B} receptor, respectively. Second, MRS 1754, which has similar affinity for the rat A_{2B} and A_1 receptors, failed to inhibit the bronchoconstrictor response to adenosine at doses which blocked the A_1 receptor-mediated bradycardia induced by NECA (Hannon et al. 2002b). Thus, the receptor(s) mediating the bronchoconstrictor response to adenosine augmented after challenge in sensitised Brown Norway rats could not be characterised as one of the four recognised adenosine receptor subtypes.

The uncertainty as to the receptor mechanism involved in the bronchoconstrictor response to adenosine plus the fact that QAF805 was a potent antagonist of the rat A_{2B} receptor led us to test QAF805 in the model. When given intravenously at a high dose of 10 mg kg^{-1}, QAF805 had no significant effects on the bronchoconstrictor response to adenosine despite causing a dose-related inhibition of the A_1 receptor mediated bradycardia induced by NECA (J.R. Fozard and L. Mazzoni, unpublished observations 2000). QAF805 was eventually tested in a Proof of Mechanism study in asthmatics as an antagonist of the bronchoconstrictor response to AMP (Pascoe et al. 2007). In a placebo-controlled, double-blind, randomized, two-way crossover trial the compound failed to block the response to AMP. Thus, from this study, neither the A_{2B} nor the A_3 receptor appears to be a major factor in the response to AMP challenge in asthmatics. However, the ability of predictive effective doses to test conclusively the hypothesis in this study can be questioned.

1.6 By What Mechanism Does Adenosine Cause Bronchoconstriction in the Rat?

Although the Brown Norway rat model did not turn out to be clinically relevant, it did reveal a fascinating pharmacological curiosity in the form of the mechanism of the bronchoconstrictor response to adenosine which could not be classified in terms of the four recognised adenosine receptor subtypes. To facilitate more precise quantitative analysis of the phenomenon, we developed an in vitro assay using parenchymal strips prepared from lungs from actively sensitised Brown Norway rats challenged with allergen. The parenchymal strip contains mast cells and a number of contractile elements of which the alveolar ducts, and smooth muscle of the small airways and in particular the pleura are the most important contributors to the contractile response (Goldie et al. 1982; Wong et al. 1992; Karmouty-Quintana et al. 2006). Whilst we did not (and still do not) know which of these tissues is responsible for the contractile response to adenosine, it was encouraging that strips from sensitised, challenged animals showed marked hyperresponsiveness to adenosine and NECA but not to CPA, CGS 21680 or 2-Cl-IB-MECA, which mimicked closely the observations in the whole animal (compare Figs. 1.4 and 1.5). Moreover,

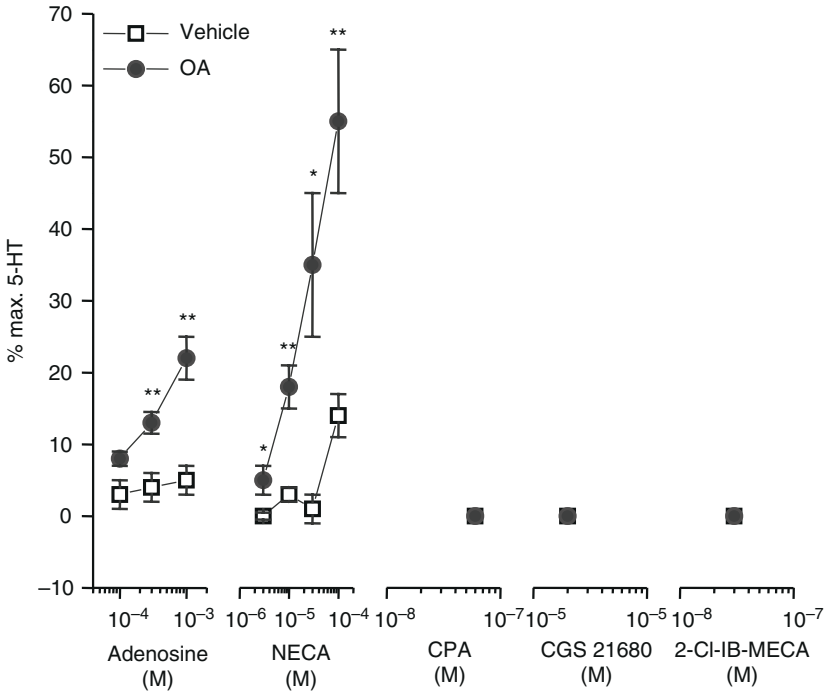

Fig. 1.5 Effects of adenosine, NECA, CPA, CGS 21680 and 2-Cl-IB-MECA on parenchymal strips prepared from lungs removed from Brown Norway rats actively sensitised to ovalbumin (OA) and challenged 3 h previously with vehicle (saline, 0.2 ml, i.t.) or OA (0.3 mg kg^{-1} i.t.). Responses are expressed relative to the response to 5-HT (10^{-4} M). Results are expressed as means ± s.e. means of between three and six individual experiments. *$P < 0.05$, **$P < 0.01$ that the value is significantly different from the equivalent value in the vehicle-challenged group (from Hannon et al. 2002b, with permission)

an initial evaluation using antagonists revealed the contractile response of the strip to be mast cell-dependent and mediated by the atypical receptor mechanism defined for the bronchoconstrictor response in vivo (Hannon et al. 2001, 2002b; Wolber et al. 2004).

1.6.1 The Use of High Concentrations of CPA Reveals a Contribution to the Contractile Response of the Parenchymal Strip to Adenosine from the A_1 Receptor

As mentioned above, a key factor in excluding a role for three out of the four recognised adenosine receptor subtypes was that the subtype selective agonists, CPA (A_1), CGS 21680 (A_{2A}) and 2-Cl-IB-MECA (A_3), unlike the non-selective agonists,

adenosine and NECA, did not induce contraction of the strips from sensitised, challenged animals despite the use of concentrations 100-fold their Ki values at the receptor sites for which they are selective (Fig. 1.5; Hannon et al. 2002b). We noted, however, that the concentrations of adenosine and NECA required to contract the strip were even higher than this. For example, we routinely used a concentration of adenosine of 1 mM which gives a contraction which is submaximal despite exceeding by 14,000-fold the Ki value at the rat A_1 receptor and by 150 times the Ki value at the rat A_3 receptor (Mueller 2003). In the case of NECA, the concentrations ranged between 3 and 100 μM which are respectively 10–300 times the Ki value at the rat A_3 receptor and 400–14,000 times the Ki value at the A_1 receptor (Fredholm et al. 2001a). On this basis, we decided to re-evaluate the effects of CPA and 2-Cl-IB-MECA at concentrations higher than those used previously.

At concentrations of 10 and 100 μM (200–2,000 times the concentration used earlier and 14,000–140,000-fold the Ki value at the rat A_1 receptor), CPA induced small contractile responses on tissues from saline-challenged animals which were increased if the strips were taken from allergen-challenged animals (Fig. 1.6).

This effect is qualitatively similar to that seen with adenosine or NECA (Fig. 1.5). The 5-HT receptor antagonist, methysergide, (which we used to define the mast

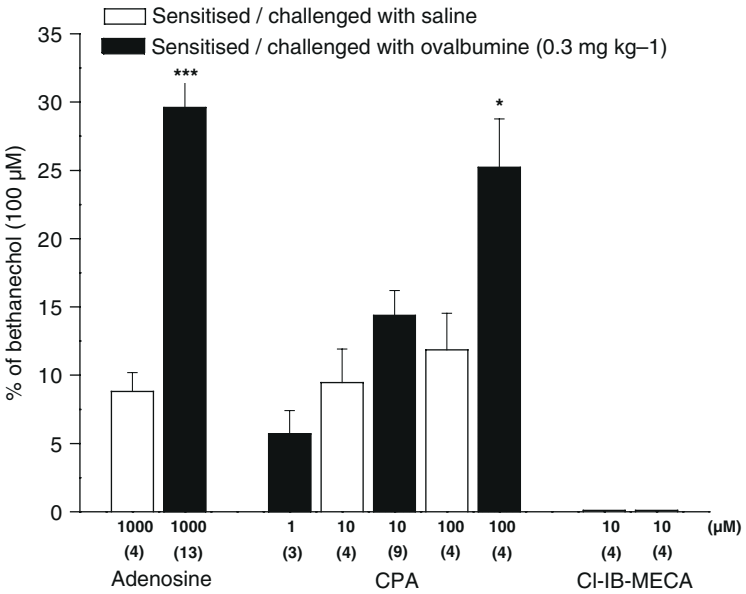

Fig. 1.6 Effect of adenosine, CPA and 2-Cl-IB-MECA at the concentrations indicated on lung parenchymal strips prepared from actively sensitised Brown Norway rats 3 h post intratracheal instillation of ovalbumin (0.3 mg kg^{-1}; *filled columns*) or saline (*open columns*). Responses are expressed relative to the response to bethanechol (100 μM) which was taken as 100%. Mean values (± SEM) from the number of individual experiments shown in parentheses are presented. * $P < 0.05$, *** $P < 0.001$ that the value differs significantly from the equivalent value in the saline challenged group (from Wolber and Fozard 2005, with permission)

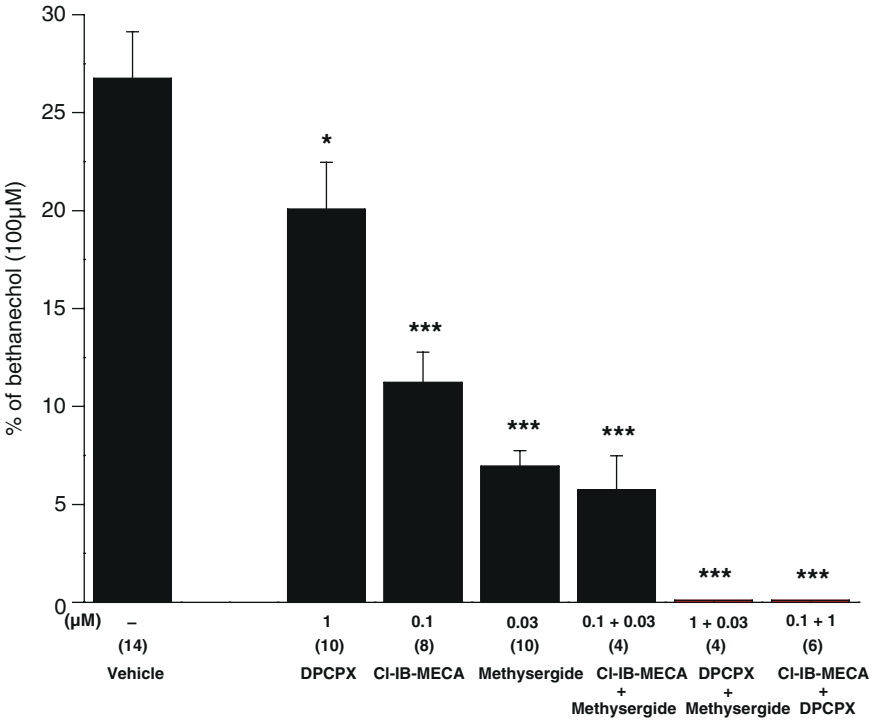

Fig. 1.7 Effect of DPCPX, 2-Cl-IB-MECA and methysergide alone or in combination at the concentrations indicated on the response to adenosine (1 mM) on lung parenchymal strips prepared from actively sensitised Brown Norway rats challenged 3 h previously with ovalbumin (0.3 mg kg^{-1} intratracheally). Responses are expressed relative to the response to bethanechol (100 µM) which was taken as 100%. Mean values (±SEM) from the number of individual experiments shown in parentheses are presented. * $P < 0.05$, *** $P < 0.001$ that the value differs significantly from the value in the vehicle group group (from Wolber and Fozard 2005, with permission)

cell-mediated component of the contractile response – for justification see Hannon et al. 2001) and the selective A_1 receptor antagonist, DPCPX, each partly blocked the augmented response to CPA and in combination abolished the response (Wolber and Fozard 2005). Thus, the response to a very high concentration of CPA augmented following allergen challenge, is mediated in part by an A_1 receptor (which is a minor component) and in part by a non-A_1 receptor mechanism that is sensitive to methysergide and hence mast cell mediated. A similar analysis of the response to a submaximal concentration of adenosine also indicated mediation in part by the A_1 receptor (ca. 20%) and in part by a mast cell dependent mechanism (ca. 80%) (Fig. 1.7). Support for this interpretation came from an analysis of five antagonists with A_1 receptor blocking activity but with no activity at the A_3 receptor at the concentrations used (8-SPT, CGS 15943, XAC (xanthine amine congener;

8-[4-[[[[(2-aminoethyl)amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine, DPCPX (1,3-dipropyl-8-cyclopentylxanthine) and MRS 1754 (*N*-4-cyanophenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1*H*-purin-8yl) phenoxy]acetamide)). Within the limits of experimental variation ca. 80% of the response to adenosine was resistant to blockade by each of these compounds (Wolber and Fozard 2005).

1.6.2 2-Cl-IB-MECA Is a Silent Antagonist of the Mast Cell-Dependent Component of the Response to Adenosine and Reveals a Contribution to the Contractile Response from the A_3 Receptor

An abundant literature implicates the A_3 receptor in the activation/degranulation of rat mast cells induced by adenosine receptor agonists (see Fozard and Hannon 2000). It was therefore surprising that ultra-high concentrations (30,000-fold the Ki value at the rat A_3 receptor) of the potent, selective A_3 receptor agonist, 2-Cl-IB-MECA, did not contract the parenchymal strip taken from sensitized challenged animals (Fig. 1.5), whereas the weaker but selective A_3 receptor agonist, inosine (Jin et al. 1997; Tilley et al. 2000; Fredholm et al. 2001b; Gao et al. 2001), did (Wolber and Fozard 2005). (We were careful to verify that the contractile response to inosine was mediated largely by 5-HT released from mast cells as a result of activation of A_3 receptors – Wolber and Fozard 2005).

This result led us to consider the possibility that 2-Cl-IB-MECA might be an antagonist at the site (probably the A_3 receptor) mediating the mast cell-dependent contractile response to adenosine. Although from the literature 2-Cl-IB-MECA behaves in most tissues as a full agonist at the A_3 receptor (the intrinsic efficacy of Cl-IB-MECA at the human and rat A_3 receptors has been reported to be ≥99% (Gao et al. 2003; Gao and Jacobson 2004)), there are occasional examples of 2-Cl-IB-MECA behaving as a partial agonist. For instance, Fossetta et al. (2003) showed 2-Cl-IB-MECA to be a low efficacy partial agonist (maximum effect 25–33% of that of adenosine) with respect to calcium signalling in human monocyte-derived dendritic cells and recombinant HEK 293 cells expressing the human A_3 receptor and a chimeric Gαq-i3 protein. Further, in CHO cells engineered to express the human A_3 receptor, 2-Cl-IB-MECA was a full agonist in arrestin translocation and in a cAMP assay but was a partial agonist for calcium accumulation (maximum effect ca. 60% that of NECA used as a full agonist – Gao and Jacobson 2008). Moreover, a number of close analogues of 2-Cl-IB-MECA behave as low efficacy partial agonists at both rat and human A_3 receptors (Gao et al. 2002, 2003; Gao and Jacobson 2004). We therefore tested 2-Cl-IB-MECA for antagonist activity against the natural ligand, adenosine, which we assumed to be a full agonist. Once again the A_3 receptor showed its capacity for surprise as 2-Cl-IB-MECA proved to be a potent, entirely silent antagonist of the contractile response to adenosine (threshold for blockade ≥10 nM; maximum blockade ≤100 nM), although predictably, as explained below, blockade was incomplete (Fig. 1.8).

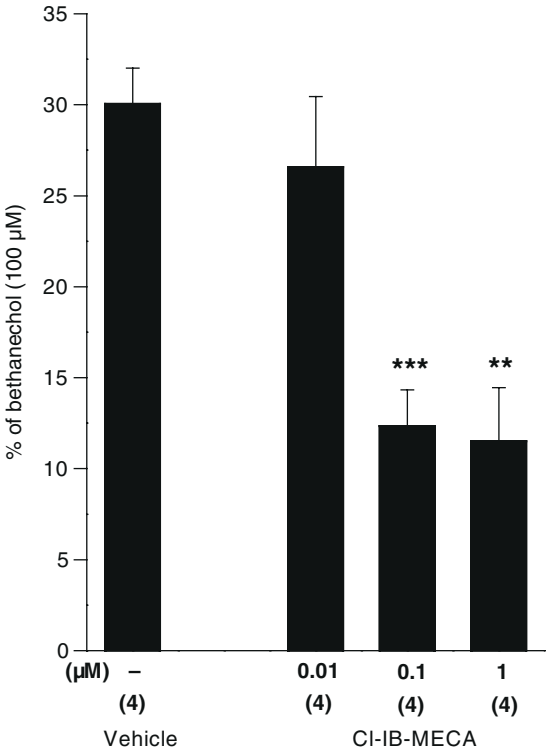

Fig. 1.8 Effect of 2-Cl-IB-MECA at the concentrations indicated on the response to adenosine (1 mM) on lung parenchymal strips prepared from actively sensitised Brown Norway rats challenged 3 h previously with ovalbumin (0.3 mg kg^{-1} intratracheally). Responses are expressed relative to the response to bethanechol (100 µM) which was taken as 100%. Mean values (± SEM) from the number of individual experiments shown in parentheses are presented. ** $P < 0.01$, *** $P < 0.001$ that the value differs significantly from the value in the vehicle group (from Wolber and Fozard (2005) with permission)

Since combining 2-Cl-IB-MECA with methysergide induced no further blockade than was seen with either substance given alone (Fig. 1.7) it can be concluded that 2-Cl-IB-MECA and methysergide act on the same component in the response to adenosine which is mast cell mediated. Moreover, selective blockade of adenosine by 2-Cl-IB-MECA at low concentrations would be consistent with the A_3 receptor being involved in the response. The finding is to my knowledge the first demonstration of 2-Cl-IB-MECA behaving as a silent antagonist at the A_3 receptor. Co-administration of 2-Cl-IB-MECA with DPCPX abolished the response to adenosine (Fig. 1.7) which indicates that 2-Cl-IB-MECA also acts on an A_1 receptor which is not mast cell located.

A further piece of evidence consistent with a role for A_1/A_3 receptors is that the augmented response to adenosine was abolished when parenchymal strips were prepared from lungs removed from pertussis toxin-treated animals which had been

challenged with antigen. Pertussis toxin-sensitive G proteins include the Gi and Go families and are involved in the signalling pathways linked to A_1 and A_3 receptors (Fredholm et al. 2001a; Zhong et al. 2003).

But with adenosine A_3 receptor pharmacology it seems that nothing is entirely straightforward and the site had one further surprise in store. Thus, we tested the selective, but relatively weak, rat A_3 receptor antagonists, MRS 1523 (2,3-diethyl-4, 5-dipropyl-6-phenylpiridine4-3-thiocarboxylate-5-carboxylate) and MRS 1191 (3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate), against the adenosine contractile response on the strip. Surprisingly, these compounds showed no antagonist effects towards adenosine despite the use of concentrations up to 30- and sevenfold their affinities for the A_3 receptor, respectively. From the literature, similar or lower concentrations of MRS 1523 and MRS 1191 have been shown to be effective in blocking a variety of responses mediated through the rat or human A_3 receptor (see for example, Shneyvays et al. 2000; Ezeamuzie and Philips 2003; Hentschel et al. 2003; Hinschen et al. 2003). However, there is at least one report which is in agreement with our observations: Thus, inhibition of human thyroid cancer cell proliferation induced by admittedly high concentrations of 2-Cl-IB-MECA were also resistant to blockade by MRS 1523 or MRS 1191 (Morello et al. 2008).

Thus, at this stage, we have clarified, but not fully explained, the receptor mechanism which mediates the adenosine-induced contraction of the parenchymal strip prepared from the lungs of actively sensitised Brown Norway rats challenged with allergen. The response arises from activation of two Gi-protein coupled receptors. One is the A_1 receptor which is not mast cell located and hence probably on the smooth muscle of one or more of the contractile elements of the strip referred to earlier: It contributes to only a minor extent to the contractile response under the conditions of our experiments. The second is a receptor which is present on the mast cells of the lung and which shows similarities to the rat A_3 receptor in its agonist and antagonist pharmacology and in particular that Cl-IB-MECA behaves as a potent silent antagonist. However, two selective A_3 receptor antagonists, MRS 1523 and MRS 1191, are inactive at concentrations which substantially exceed their affinities for the rat A_3 receptor.

1.6.3 Does the Mechanism of the Contractile Response on the Parenchymal Strip Explain the Bronchoconstrictor Response to Adenosine in the Whole Animal?

The answer is, not exactly, although there are many similarities.

In both cases the response to adenosine is mainly mast cell mediated. Indeed, at the doses of adenosine used the contribution of the A_1 receptor (which is not mast cell mediated) to the bronchoconstrictor response in vivo is minimal (Hannon et al. 2002b). The pharmacological analysis gave generally similar results although

because of the difficulty in giving high doses of the agonist and antagonist ligands in vivo without affecting adversely the viability of the preparations, the analysis is necessarily restricted. The one major difference is that 8-SPT blocked dose-dependently the bronchoconstrictor response to adenosine in vivo (Hannon et al. 2002) but even at high concentrations had no blocking activity on the mast cell-mediated component of the contractile response to adenosine on the strip (Wolber and Fozard 2005).

1.7 Conclusion

My personal interaction with the A_3 receptor ended in 2005 when I retired from full time pharmacology. The interaction led me through basic and applied pharmacology and resulted in a fascinating insight into one small part of the biological significance of this receptor. My chemistry colleagues discovered impressive molecules with the potential for use as pharmacological tools and, in the case of QAF805, for a Proof of Mechanism clinical evaluation. As is usual in these situations, there remain a number of unanswered questions which will have to be explored by others. For instance, time ran out before I could test for antagonist activity of 2-Cl-IB-MECA against adenosine in vivo. Also, I have no explanation for why the agonist concentrations needed to activate the contractile mechanisms in the strip are so high. Why the blocking activity of 8-SPT against adenosine is seen in vivo but not in vitro remains a mystery. And there are others. As I hope to have conveyed in this article, at times my interaction with the A_3 receptor has been a frustrating experience, at times rewarding always challenging but never uninteresting. A generation has now passed since the A_3 receptor was first described and there exists an immense literature associated with the site. Despite this, much remains to be clarified with respect to the biological role of the A_3 receptor if we are to capitalise on the potential of the site to provide novel therapeutic strategies The work described in this volume will prepare the ground for the next generation of scientists prepared to exchange a modicum of frustration for the occasional reward and a guaranteed high level of scientific interest.

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Part II
Physico-chemical Properties and Molecular Biology

Chapter 2
Thermodynamic Analysis in Drug–Receptor Binding: The A_3 Adenosine Receptor

Pier Andrea Borea, Stefania Gessi, Stefania Merighi, and Katia Varani

2.1 Introduction

Adenosine is identified as a major local regulator of tissue functions and mediates its effects by interaction with four cell surface receptor subtypes named as A_1, A_{2A}, A_{2B} and A_3 adenosine receptors which are widely distributed through the body (Baraldi et al. 2008). They are coupled to different G proteins: A_1 and A_3 (via Gi) mediate inhibition of the adenylate cyclase activity, whereas both A_{2A} and A_{2B} (via Gs) subtypes stimulate cAMP accumulation (Fredholm et al. 2001; Burnstock 2006). Among the four adenosine subtypes the A_3 is the latest cloned and pharmacologically characterized: the amino acid sequence of the human A_3 receptors is 49.5%, 43.2% and 39.9% identical in sequence to human A_1, A_{2A} and A_{2B} receptors, respectively. Among the various species, rat A_3 receptors is significantly different from human having 73.8% of identical sequence whereas 85.2% homology is shown between sheep and human A_3 receptors (Martinelli and Tuccinardi 2008). The presence of A_3 receptors has been evaluated by using radioligand binding, mRNA, western blotting analysis or functional assays in a variety of primary cell cultures, native tissues and cell lines (Gessi et al. 2008a). It is well accepted that A_3 adenosine receptors are primarily expressed in lung, kidney, heart, brain, liver and colon (Varani et al. 2006; Burnstock 2007; Gessi et al. 2007). So, their modulation by using agonists or antagonists represents an interesting pharmacological tool in a variety of diseases (Baraldi et al. 2008). At the present, a number of novel agonists and antagonists are in biological testing for several clinical indications suggesting that A_3 adenosine receptors may play a basic pharmacological role. A body of evidence from extensive biological research suggests the difficulties to identify new

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specific adenosine compounds with high affinity, selectivity and potency. The only method practicable to quantify the affinity of novel compounds is that based on binding constant measurements which allows the determination of drug–receptor affinity constants, K_A (Raffa and Porreca 1989). This specific experimental approach gives quantitative parameters linked to the capability of the drug to interact with a given receptor but provides little information on the molecular mechanisms underlying the interaction itself (Holdgate and Ward 2005). Due to this contribution it is of interest to obtain determinations of drug–receptor binding association (K_A) and dissociation ($1/K_A = K_D$) constants over a range of temperatures. This adds significant informations on the molecular mechanisms involved in the drug–receptor interaction in contrast to the single point temperature assays (Raffa 2001a). In fact, simple determination of K_A (or K_D) values makes it possible to calculate the standard free energy $\Delta G° = -RT\ lnK_A = RTlnK_D$ (T = 298.15 K) but not its two components, the equilibrium standard enthalpy ($\Delta H°$) and entropy ($\Delta S°$), as defined by the Gibbs equation $\Delta G° = \Delta H° - T\Delta S°$ (Raffa 2001b). The concentrations of membrane receptors present in biological tissues are extremely low, typically in the range from 1 to 100 fmol/mg protein for most neurotransmitter receptors and this makes impossible any microcalorimetric determination of the enthalpy (Bylund and Yamamura 1990; Ladbury 2004). As a consequence, a method based on K_D measurements over a range of temperatures combined with van't Hoff plot analysis has been successfully applied to different receptor systems to obtain the thermodynamic terms of Gibbs equation (Borea et al. 2000). The analysis of thermodynamic data of drug–receptor interaction offers an effective tool for investigating at a molecular level the role played during the binding of ligand substituents and of receptor amino acids. It can be assumed that standard enthalpy is a quantitative indicator of the changes in intermolecular bond energies such as hydrogen bonding and van der Waals interactions occurring during the binding. Standard entropy, on converse, can be considered an indicator of the rearrangements undergone by the solvent normally water molecules during the same process (Gilli and Borea 1991; Raffa 2001a, b). In the last few years it has been reported that enthalpy and entropy values of drug interaction with a defined receptor can often give a simple way to discriminate the effect mediated by the ligands. This particular property has been called "thermodynamic discrimination" and results from the fact that the binding of agonists may be entropy and that of antagonists enthalpy-driven, or vice versa (Borea et al. 2000). At the present 16 membrane receptor systems have been extensively studied from a thermodynamic point of view: ten of these show the agonist–antagonist discrimination and six are not discriminated.

The first case of thermodynamic discrimination was reported for the β adrenergic receptors where agonists have H-driven binding and antagonists have H&S-driven binding (Weiland et al. 1979). Recently, the discovery of thermodynamic discrimination of the histamine H_3 receptors suggests that agonist binding is entropy-driven and the antagonist binding is enthalpy-driven (Harper and Black 2007). This phenomenon has not been observed for five other G-protein coupled receptors (GPCR) such as D_2-dopamine, serotonin $5HT_{1A}$, opioid and cholecystokinin

receptors (Duarte et al. 1988; Dalpiaz et al. 1996; Borea et al. 1988; Harper et al. 2008). As for the membrane receptors it has been demonstrated that A_1, A_{2A}, A_{2B} and A_3 adenosine receptors are thermodynamically discriminated and agonist binding is entropy-driven and antagonists have enthalpy–entropy driven binding (Borea et al. 1994, 1996a, 2001; Gessi et al. 2008b; Merighi et al. 2002; Varani et al. 2000, 2008a). In addition, five out of six ligand-gated ion channel receptors (LGICR), that is glycine, GABA, serotonin $5HT_3$, neuronal nicotinic and purinergic $P2X_3$ receptors discriminate in vitro agonists from antagonists (Gomez et al. 1989; Maksai 1994; Dalpiaz et al. 1995; Borea et al. 1996b, 1998, 2004; Varani et al. 2008b, c). On the contrary only the purinergic $P2X_1$ receptors do not show any discrimination between agonists and antagonists both characterised by enthalpy and entropy-driven binding (Varani et al. 2008b, c). Furthermore thermodynamic discrimination could be very useful for a deeper antagonist characterization. As a matter of fact a large number of antagonists of several membrane receptors have been recognized as inverse agonists indicating neutral antagonists as minority species in the pharmacological field (Kenakin 2004; Gilli et al. 2005). Finally, the analysis of the thermodynamic parameters for agonists suggests that the affinity and efficacy of the ligands could be related. In particular, a statistically significant correlation has been verified between standard entropy and intrinsic activity values of A_1 adenosine ligands confirming that, in this case, the molecular factors determining the recognition process (receptor binding affinity) are the same as those involved in the signal transduction mechanisms (intrinsic activity). This observation could allow to discriminate between partial and full agonists by using standard entropy values that appear to be the most relevant physicochemical parameters (Borea et al. 1994, 2000). Another significant thermodynamic aspect, which characterizes the membrane receptors is the linearity of the van't Hoff plots in the temperature range usually investigated (0–35°C) indicating that the heat capacity value ($\Delta Cp°$) of the binding equilibrium is nearly zero, a phenomenon which is not usual in reactions involving biomacromolecules in solution (Sturtevant 1977). For this reason both ΔH and ΔS values are independent of temperature and their standard values can be obtained by linear van't Hoff plots. At the present only two examples are reported in literature showing non linear van't Hoff plots and negative $\Delta Cp°$ values: the thrombin receptor (PAR-1) and the cytoplasmic steroid nuclear receptors where there are relevant intramolecular hydrophobic contributions to the binding (Boysen et al. 2002; Gilli et al. 2005). Because of the magnitude of this phenomenon the molecular characteristics of the ligands and the determination of the $\Delta Cp°$ allows comparative assessment of analogs which can be structurally rationalized in respect of their ligand-binding behaviour (Gilli and Borea 1991). Analysis of enthalpy and entropy parameters also shows the very strict intercorrelation of ΔH and ΔS values of receptor binding, a general phenomenon called "thermodynamic compensation". The solvent molecules do not affect the affinity constant (K_A) of the drug–receptor interaction because the standard free energy for solvent reorganization can be shown to average to zero and therefore the values of the binding constants are most probably determined by the specific features of the ligand and receptor undergoing the binding process, irrespectively of the solvent

(Tomlinson 1983; Gilli et al. 1994; Grunwald and Steel 1995; Borea et al. 2004). Conversely, the interrelated enthalpy and entropy values seem to be mostly related to the rearrangements occurring during the binding in the solvent or better at both solvent–drug and solvent–receptor interfaces (Grunwald and Steel 1995). Recently it has been reported that the desolvation of ligands from surrounding water molecules and the resulting rearrangement of the hydrogen bonding network may actually be the primary driving force for determining ligand binding affinities in the absence of specific functional group interactions (Leung et al. 2008).

2.2 Methods of Thermodynamic Measurement of Drug–Receptor Interaction

There are two main strategies for the evaluation of the standard free energy ($\Delta G°$), standard enthalpy ($\Delta H°$) and standard entropy ($\Delta S°$) terms. The first consists in determining equilibrium constants $\Delta G° = -RT\ln K_A$ in association with direct microcalorimetric enthalpy measurements, even if this method is not practicable in binding studies for the very low receptor concentration in most tissues. The only method of practical use consists in measurements of K_A carried out at different temperatures followed by van t' Hoff analysis. Such a method has proved to be successful in many cases and has been applied to the study of several GPCR membrane receptors such as β-adrenoceptor, serotonin $5HT_1$, μ, δ, κ opioid and nociceptin, dopamine D_2, histamine H_3 and cholecystokinin CCK_2 receptors (Weiland et al. 1979; Dalpiaz et al. 1996; Borea et al. 1988; Varani et al. 1998; Duarte et al. 1988; Harper and Black 2007; Harper et al. 2008). Similarly also A_1, A_{2A}, A_{2B} and A_3 adenosine receptors have been thermodynamically studied (Borea et al. 1994, 1996a; Gessi et al. 2008b; Merighi et al. 2002; Varani et al. 2000, 2008a). Thermodynamic analysis has been also evaluated for LGICR i.e. glycine, $GABA_A$, serotonin $5HT_3$, nicotinic, $P2X_1$ and $P2X_3$ purinergic receptors (Gomez et al. 1989; Maksai 1994; Borea et al. 1996b, 2004; Varani et al. 2008b). In addition the evaluation of the binding thermodynamic parameters has also been used for cytoplasmic steroid/nuclear receptors such as estrogen, progesterone and androgen subtypes (Gilli et al. 2005).

The general approaches used for affinity constant determination and for evaluation of thermodynamic parameters will be described.

2.3 Affinity Constant Determination

For a generic binding equilibrium L + R = LR (L = ligand, R = receptor) the affinity association constant is calculated as $K_A = [LR]/[L][R] = [LR]/[Lmax - LR][Bmax - LR] = 1/K_D$, where [Lmax] = total concentration of ligand added, [Bmax] = total concentration of binding sites, and K_D = dissociation constant. As $[LR]/[Lmax - LR] = [Bound]/[Free] = [Bmax]\ K_A - K_A\ [Bound]$, the K_A and Bmax values can be

obtained in saturation binding experiments, from the slope and intercept of the Scatchard plot [Bound]/[Free] versus [Bound]. In competition binding experiments, Ki values can be calculated from IC_{50} values according to the equation: $Ki = IC_{50}/(1 + [C^*]/K_D^*)$ where [C*] is the concentration of the radioligand added, and K_D^* its dissociation constant (Cheng and Prusoff 1973). The LIGAND weighted non linear least-square curve fitting program (Munson and Rodbard 1980) was used for computer analysis of saturation and competition experiments which are usually performed in the temperature 0–35°C.

2.4 Thermodynamic Parameters Determination

Measurement of K_A values (apparent association constant of the ligand) at different temperatures allow to obtain the equilibrium thermodynamic parameters such as the standard free energy $\Delta G° = -RT\ln K_A$ (where T is the temperature in kelvin = 298.15 K and R is the ideal gas constant = 8.314 J/K/mol) which can be separated in its enthalpic and entropic contributions according to the Gibbs equation: $\Delta G° = \Delta H° - T\Delta S°$. The standard free energy is calculated as $\Delta G° = -RT\ln K_A$ at 298.15 K. The van't Hoff plot of $\ln K_A$ versus (1/T) allows the estimation of the standard enthalpy of binding, $\Delta H°$, because the slope is $-\Delta H°/R$ and the standard entropy change, $\Delta S°$, can be estimated as the intercept $-\Delta S°/R$ or as $\Delta S° = (\Delta H° - \Delta G°)/T$. The terms $\Delta G°$, $\Delta H°$ and $\Delta S°$ indicate the measurements made under standard state conditions of 1 atm, unit activity (1 M concentration) and at 1 M hydrogen ion concentration (pH 0).

2.5 Representation of $\Delta G°$, $\Delta H°$ and $\Delta S°$ Data

Since $\Delta G°$ is related linearly to $\Delta H°$ and $\Delta S°$ by the Gibbs equation, $\Delta G° = \Delta H° - T\Delta S°$, it is useful to represent the thermodynamic data of drug–receptor interaction in a $-T\Delta S°$ versus $\Delta H°$ plot. Several advantages can be achieved by this type of representation. First of all the plot allows to obtain information on $\Delta G°$ and a consequence on K_A values ($\Delta G° = -RT \ln K_A$). In fact, the same values of $\Delta G°$ can be produced by all the linear combination of different $\Delta H°$ and $-T\Delta S°$ pairs of values lying on a diagonal of the plot. This type of representation, which will be discussed in a further section of this paper, shows immediately the presence or the absence of the thermodynamic discrimination phenomenon between agonists and antagonists for any given receptor. Finally, the thermodynamic compensation or enthalpy–entropy compensation can be evaluated by linear regression analysis of $-T\Delta S°$ versus $\Delta H°$ plots. This phenomenon will be described and discussed accurately for A_3 adenosine receptors in comparison with the data of other GPCRs and LGICRs.

2.6 Binding Thermodynamics of A_3 Adenosine Receptors

Saturation binding experiments were carried out incubating to equilibrium fractions of tissue homogenates or cell membranes with increasing concentrations of radiolabelled ligand. A_3 adenosine receptors were labelled by using different concentrations (from 0.05 to 50 nM) of [^{3}H]- 5-N-(4-methoxyphenylcarbamoyl) amino-8-propyl-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine ([^{3}H]-MRE 3008F20, specific activity 67 Ci/mmol, Amersham International Chemical Laboratories, Buckinghamshire, UK) as radioligand. Inhibition binding experiments were performed by displacing a fixed concentration of radiolabelled ligand [C*] from the receptor preparation with increasing concentrations of the unlabelled ligand under investigation with the aim of determining its IC_{50} value, that is the inhibitor concentration displacing the 50% of the labelled ligand. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass-fiber filters by using a Brandel instruments and the filter-bound radioactivity was counted by Scintillation Counter Packard TriCarb 2500 TR with an efficiency of 58%.

Table 2.1 summarizes the dissociation binding constants (K_D values) and the receptor density (Bmax values) of human A_3 adenosine receptors expressed in different cell lines at the six chosen temperatures, from 0 to 30°C. The substrates studied were CHO cells stably transfected with human A_3 adenosine receptors, Jurkat T cells, A375 and HL60 cell lines, lymphocytes and neutrophils (Varani et al. 2000; Merighi et al. 2001, 2002; Gessi et al. 2001, 2002, 2004). The same binding parameters were also calculated in bovine chondrocytes and synoviocytes as examples of peripheral native cells (Varani et al. 2008a). These values were determined at different temperatures by saturation binding experiments using the radiolabeled antagonist [^{3}H]-MRE 3008F20 which is able to label a single class of recognition sites with affinity in the nanomolar range and with different receptor densities in various substrates ranging from 76 to 1,300 fmol/mg protein. While K_D values change with temperature, Bmax values appear to be largerly independent of it suggesting a same population of receptors at all temperatures. Saturation curves and Scatchard plots obtained at the six temperature investigated for the saturation equilibrium of [^{3}H]-MRE 3008F20 revealed the presence of one class of high affinity binding sites. Figure 2.1a shows the van't Hoff plots, lnKA versus 1/T, and the temperature dependence of the affinity constants K_A in the examined cell lines. Figure 2.1b and c report the van't Hoff plots of the adenosine agonists and antagonists investigated where the affinity values were calculated in hA_3CHO cells, respectively. In the complete range of temperature, van't Hoff plots appear to be linear for the adenosine compounds, agonists or antagonists, in the different cell lines analysed. Table 2.2 reports thermodynamic parameters of [^{3}H]-MRE 3008F20 saturation binding expressed as $\Delta G°$, $\Delta H°$ and $\Delta S°$ showing, as expected, that the binding of this antagonist is enthalpy- and entropy-driven in the various cell lines investigated. In addition, Table 2.3 shows the final thermodynamic parameters of six typical adenosine agonists and five antagonists in hA_3CHO cells. $\Delta G°$ values

Table 2.1 Binding parameters expressed as dissociation constant (K_D, nM) and receptor density (Bmax, fmol/mg protein) obtained from [^{3}H]-MRE 3008F20 saturation binding experiments to different cell lines expressing A_3 adenosine receptors

[Temperatures	273.15	278.15	283.15	288.15	293.15	298.15	303.15
	K_D, nM	K_D, nM	K_D, nM	K_D, nM	K_D, nM	K_D, nM	K_D, nM
^{3}H]-MRE 3008F20 binding	Bmax, fmol/mg protein	Bmax, fmol/mg protein	Bmax, fmol/mg protein	Bmax, fmol/mg protein	Bmax, fmol/mg protein	Bmax, fmol/mg protein	Bmax, fmol/mg protein
hA_3CHO cells	0.80 ± 0.03	0.85 ± 0.02	1.20 ± 0.20	1.80 ± 0.20	2.10 ± 0.20	2.90 ± 0.10	3.30 ± 0.20
	305 ± 31	300 ± 33	310 ± 25	308 ± 28	315 ± 30	322 ± 34	320 ± 27
Jurkat T cells	1.88 ± 0.20	1.92 ± 0.21	2.52 ± 0.24	3.40 ± 0.30	4.15 ± 0.32	4.59 ± 0.37	5.33 ± 0.41
	1,300 ± 140	1,290 ± 120	1,350 ± 130	1,420 ± 160	1,360 ± 110	1,470 ± 160	1,260 ± 130
A375 cells	3.25 ± 0.37	3.30 ± 0.40	3.95 ± 0.42	4.03 ± 0.41	4.15 ± 0.44	4.36 ± 0.47	4.48 ± 0.52
	288 ± 32	291 ± 50	245 ± 30	274 ± 36	286 ± 35	337 ± 43	308 ± 32
HL60 cells	2.54 ± 0.28	2.60 ± 0.40	3.13 ± 0.28	3.40 ± 0.40	4.15 ± 0.40	4.59 ± 0.40	5.07 ± 0.53
	340 ± 28	345 ± 31	363 ± 29	314 ± 31	327 ± 31	345 ± 31	315 ± 27
Human neutrophils	2.26 ± 0.28	2.30 ± 0.30	2.65 ± 0.72	2.98 ± 0.31	3.23 ± 0.34	4.11 ± 0.44	4.41 ± 0.42
	422 ± 31	430 ± 35	428 ± 33	462 ± 35	454 ± 28	446 ± 30	434 ± 27
Human lymphocytes	1.72 ± 0.22	1.79 ± 0.30	2.84 ± 0.27	3.17 ± 0.29	3.68 ± 0.34	3.83 ± 0.32	4.45 ± 0.38
	120 ± 14	125 ± 15	122 ± 14	118 ± 14	115 ± 17	120 ± 11	124 ± 13
Bovine chondrocytes	4.55 ± 0.32	4.61 ± 0.35	5.60 ± 0.47	7.56 ± 0.71	9.24 ± 0.81	12.47 ± 0.95	15.23 ± 1.32
	76 ± 5	79 ± 6	82 ± 6	82 ± 7	85 ± 6	78 ± 7	84 ± 8
Bovine synoviocytes	1.80 ± 0.19	1.86 ± 0.22	1.94 ± 0.20	2.47 ± 0.25	3.43 ± 0.37	4.23 ± 0.43	5.38 ± 0.51
	81 ± 3	83 ± 6	77 ± 4	78 ± 4	80 ± 4	87 ± 4	85±4

[^{3}H]-MRE 3008F20 saturation binding experiments were performed at different temperatures from 0°C to 30°C. Values are expressed as means ± SEM.

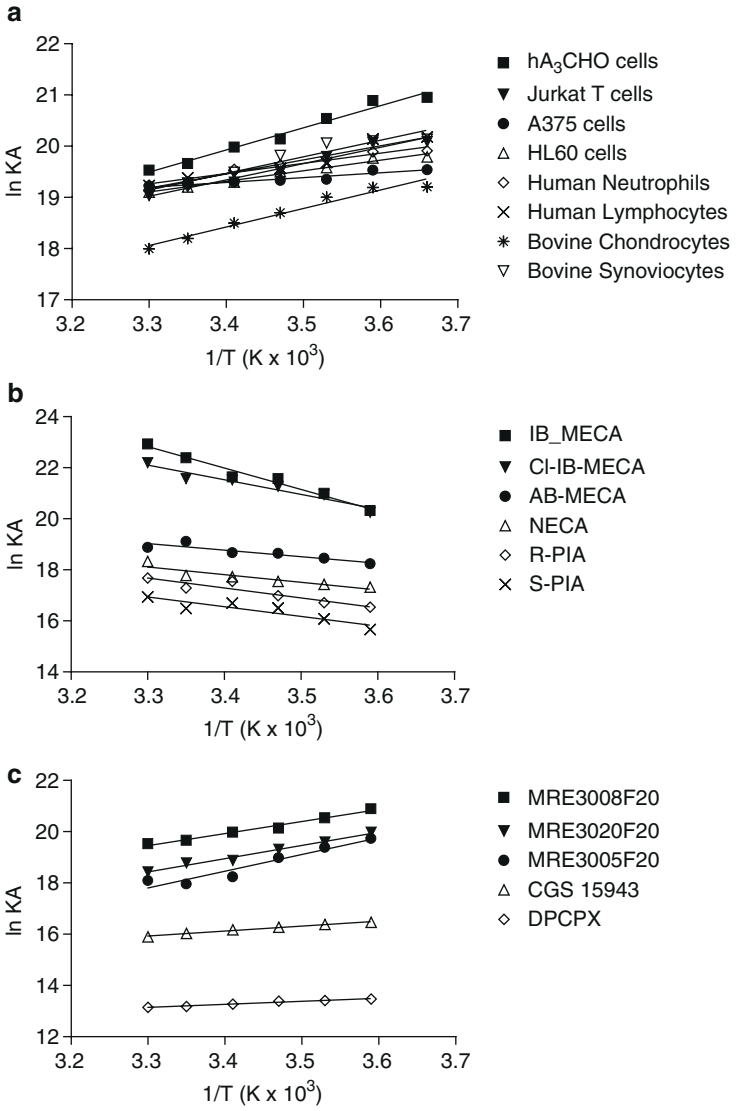

Fig. 2.1 van't Hoff plots showing the effect of temperature on the equilibrium binding association constants, K_A, for [^{3}H]-MRE 3008F20 in various cell lines examined (**a**) and for six adenosine receptor agonists (**b**) and antagonists (**c**) studied. All plots are essentially linear (r in the range 0.91–0.99) in the temperature range of 0–30°C

range from −55.2 to −41.3 kJ/mol for agonists and from −48.8 to −32.6 kJ/mol for antagonists. Equilibrium standard enthalpy (ΔH°) and entropy (ΔS°) values suggest that the binding of agonists is always totally entropy-driven while it is enthalpy- and entropy-driven for antagonists. In particular, ΔH° values range from 21 to 67

Table 2.2 Thermodynamic parameters for [^{3}H]-MRE 3008F20 saturation binding experiments to different cell lines expressing A_3 adenosine receptors

	ΔG°	ΔH°	ΔS°
[^{3}H]-MRE 3008F20 binding	kJ/mol	kJ/mol	J/mol/K
hA_3CHO cells	−48.55 ± 0.15	−33.11 ± 3.13	52.25 ± 5.53
Jurkat T cells	−47.31 ± 0.14	−27.97 ± 3.18	64.89 ± 6.35
A375 cells	−47.60 ± 0.13	−7.62 ± 1.11	134.2 ± 10.6
HL60 cells	−47.43 ± 0.16	−17.71 ± 1.65	99.73 ± 9.05
Human neutrophils	−47.74 ± 0.13	−18.53 ± 1.15	98.07 ± 9.25
Human lymphocytes	−47.76 ± 0.16	−19.78 ± 1.50	94.07 ± 7.11
Bovine chondrocytes	−47.80 ± 0.16	−33.93 ± 3.42	44.48 ± 4.25
Bovine synoviocytes	−48.12 ± 0.14	−35.59 ± 3.67	42.08 ± 4.15

Thermodynamic parameters ΔG°, ΔH° and ΔS° are expressed as means ± SEM, temperature used was 25°C.

Table 2.3 Thermodynamic parameters for [^{3}H]-MRE 3008F20 displacement binding experiments to hA_3CHO cells

	ΔG°	ΔH°	ΔS°
[^{3}H]-MRE 3008F20 binding	kJ/mol	kJ/mol	J/mol/K
Agonists			
IB-MECA	−55.20 ± 0.20	67.03 ± 4.86	410 ± 8
Cl-IB-MECA	−53.81 ± 0.11	46.07 ± 3.02	355 ± 5
AB-MECA	−46.62 ± 0.21	21.05 ± 1.04	225 ± 4
NECA	−44.32 ± 0.13	24.06 ± 2.08	228 ± 9
R-PIA	−43.14 ± 0.22	31.12 ± 2.05	248 ± 7
S-PIA	−41.31 ± 0.33	30.05 ± 1.27	241 ± 5
Antagonists			
MRE 3008F20	−48.72 ± 0.18	−36.72 ± 3.26	48.24 ± 4.12
MRE 3020F20	−46.21 ± 0.11	−41.12 ± 2.02	16.23 ± 2.14
MRE 3005F20	−44.81 ± 0.22	−52.27 ± 2.38	24.13 ± 2.13
CGS 15943	−39.62 ± 0.12	−15.47 ± 1.45	81.14 ± 6.15
DPCPX	−32.63 ± 0.23	−9.42 ± 0.91	78.25 ± 8.11

Thermodynamic parameters ΔG°, ΔH° and ΔS° are expressed as means ± SEM, temperature used was 25°C.

and from −52 to −9 kJ/mol and ΔS° values from 225 to 410 J/K/mol and from 16 to 81 J/K/mol for agonists and antagonists, respectively. Figure 2.2a shows the results in the form of −TΔS° versus ΔH° scatter plot (T = 298.15 K). It becomes apparent that all points are arranged on the same diagonal band encompassed between the two dashed lines which represent the loci of points defined by the limiting K_D values of 100 μM and 10 pM. This band is the expression of the enthalpy–entropy (E–E) compensation phenomenon. Final thermodynamic parameters of the different compounds investigated are reported in Table 2.3. All these

data demonstrate that adenosine agonists and antagonists are thermodynamically discriminated. Figure 2.2b summarizes in the form of $-T\Delta S°$ versus $\Delta H°$ scatter plot the results of the thermodynamic parameters of A_1, A_{2A}, A_{2B} and A_3 adenosine receptor agonists and antagonists.

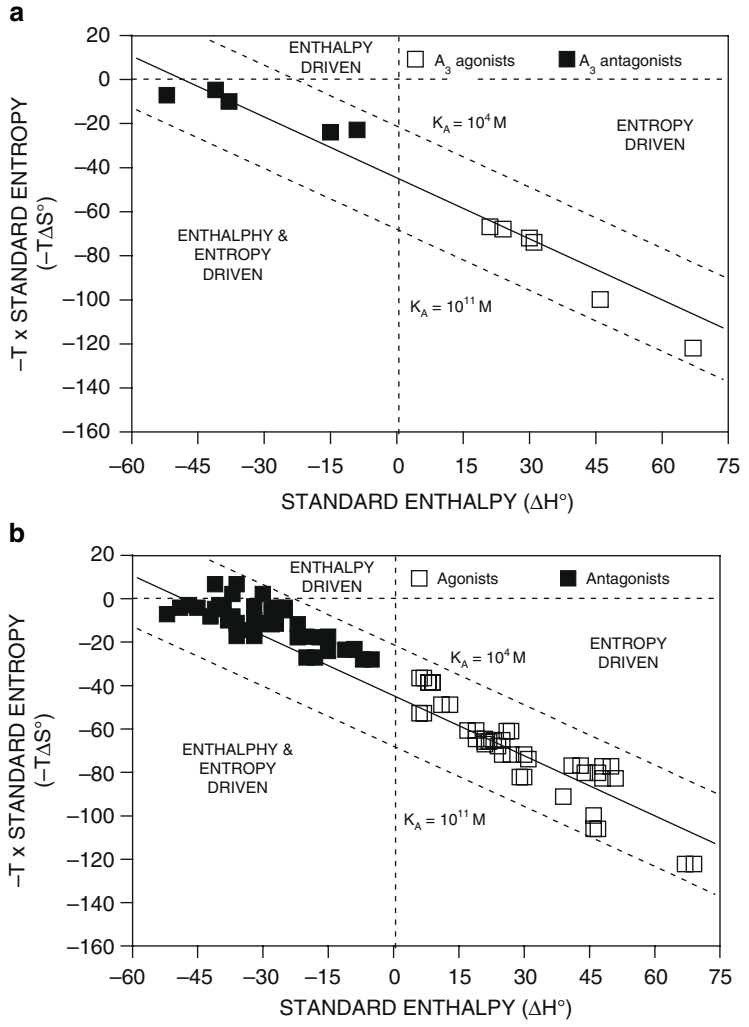

Fig. 2.2 Scatter plot of $-T\Delta S°$ versus $\Delta H°$ values for the adenosine A_3 receptor ligands (n = 11) studied obtained in hA_3 CHO cells (**a**). Scatter plot of a typical series of adenosine compounds (n = 85) for the A_1, A_{2A}, A_{2B} and A_3 adenosine receptor subtypes (**b**). *Full* and *open symbols* indicate antagonists and agonists, respectively. All points lie on a same regression line. The two *dashed lines* indicate the loci of the points representing possible combinations of $\Delta H°$ and $-T\Delta S°$ values giving rise to the two different equilibrium constants indicated ($K_A = 10^4$ and $K_A = 10^{11}$ M^{-1})

2.7 Binding Thermodynamics of G-Protein Coupled Receptors

Table 2.4 and Fig. 2.3a summarize the thermodynamic parameters of G-protein coupled receptors (GPCRs) so far studied where the ranges of $\Delta G°$, $\Delta H°$ and $\Delta S°$ for both agonist and antagonist binding (n = 203) are given together with a qualitative classification of the equilibrium driving force. The analysis of the data revealed that six out of the ten GPCRs reported are discriminated. For dopamine D_2 receptor thermodynamic values for antagonist ($-89 \leq \Delta H° \leq 59$ kJ/mol; $-105 \leq -T\Delta S° \leq 107$ kJ/mol/K) and agonist binding ($-224 \leq \Delta H° \leq 90$ kJ/mol; $-136 \leq -T\Delta S° \leq 176$ kJ/mol/K) are scattered over their complete range. Therefore, agonists and antagonists do not show thermodynamic discrimination (Duarte et al. 1988). A similar behaviour is shown by the $5HT_{1A}$ receptors where antagonist ($15 \leq \Delta H° \leq 80$ kJ/mol; $-109 \leq -T\Delta S° \leq -47$ kJ/mol/K) and agonist binding ($-65 \leq \Delta H° \leq 58$ kJ/mol; $-109 \leq -T\Delta S° \leq 20$ kJ/mol/K) do not suggest any agonist-antagonist discrimination (Dalpiaz et al. 1996). As for opioid receptors, antagonists ($-52 \leq \Delta H° \leq 5$ kJ/mol; $-15 \leq -T\Delta S° \leq -2$ kJ/mol/K) and agonists ($-42 \leq \Delta H° \leq 12$ kJ/mol; $-19 \leq -T\Delta S° \leq -4$ kJ/mol/K) are not thermodynamically discriminated (Borea et al. 1988; Li et al. 1998). This result is in qualitative agreement with that reported for the binding of nociceptin receptors where the agonist binding was entropy-driven (Varani et al. 1998). Recently, in light of the potential for radioligand thermodynamic studies, the cholecystokinin CCK_2 receptors have been investigated to verify the discrimination of agonists and antagonists. The finding of a lack of thermodynamic discrimination between agonists and antagonists at the CCK_2 receptors has been explained by suggesting that small molecules may each have a unique combination of individual interactions with the receptors (Harper et al. 2007a, 2008). As for the β-adrenergic receptor, agonist cluster in the exothermic region ($-143 \leq \Delta H° \leq -17$ kJ/mol) with negative or weakly positive standard entropy values ($-8 \leq -T\Delta S° \leq 93$ kJ/mol/K). Agonist binding is therefore to be classified as enthalpy-driven. Conversely, the antagonist binding is mostly or totally entropy-driven ($-21 \leq \Delta H° \leq 16$ kJ/mol; $-53 \leq -T\Delta S° \leq -16$ kJ/mol/K) (Weiland et al. 1979). Recently, the finding that histamine H_3-receptor agonist binding was entropy-driven was explained by the disorganization of a solvation sphere around the ligands as they bind to the receptor (Harper et al. 2007b; Harper and Black 2008). Another possible explanation suggested was that the agonist binding at histamine H_3-receptors induces ternary complex formation (ARG or AR*G) and this brings to the large increase in entropy. Interestingly, the presence of salts such as $CaCl_2$ in the buffer solution changes the thermodynamic behavior of histamine ligands. In these experimental conditions agonists and antagonists showed similar thermodynamic parameters. This may be a consequence of the capability of buffer salts to increase the hydration of the ligands so that more water has to be removed during the receptor binding interaction (Harper and Black 2008). Thermodynamic parameters were also reported for A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. Agonist binding at the A_1 adenosine receptors can be classified as totally entropy-driven ($9 \leq \Delta H° \leq 50$ kJ/mol; $-106 \leq -T\Delta S° \leq -61$ kJ/mol), while antagonist binding is enthalpy- and entropy-driven ($-44 \leq \Delta H° \leq -12$ kJ/mol; $-18 \leq -T\Delta S° \leq 7$ kJ/mol/K) (Borea et al. 1994; Lorenzen et al. 2000). As for the A_{2A} adenosine

Table 2.4 Thermodynamic parameters, ΔG°, ΔH° and ΔS° of a series of typical ligands to eleven G-protein coupled receptors so far studied

		ΔG°	ΔH°	ΔS°		
GPCRs	N	kJ/mol	kJ/mol	J/mol/K	EDF	References
Dopamine D_2						Duarte et al. 1988
Agonists	11	−53 to −34	−224 to 90	−590 to 456	ND	
Antagonists	22	−59 to −24	−89 to 59	−359 to 352	ND	
Serotonin $5HT_{1A}$						Dalpiaz et al. 1996
Agonists	8	−58 to −36	−65 to 58	−67 to 366	ND	
Antagonists	7	−49 to −29	15 to 80	158 to 366	ND	
Opioid						Borea et al. 1988
Agonists	9	−63 to −47	−42 to 12	13 to 64	ND	
Antagonists	6	−59 to −50	−52 to 5	5 to 49	ND	
Cholecystokinin CCK_2						Harper et al. 2008
Agonists	2	−47 to −49	−71 to −64	−74 to −58	ND	
Antagonists	6	−51 to −36	−65 to −3.5	−67 to 152	ND	
β Adrenoceptors						Weiland et al. 1979
Agonists	14	−51 to −26	−143 to −17	−312 to 27	H-driven	
Antagonists	23	−61 to −31	−21 to 16	54 to 178	H&S-driven	
Histamine H_3						Harper and Black 2007
Agonists	7	−58 to −48	−31 to −23	198 to 311	S-driven	
Antagonists	3	−55 to −47	6 to 45	57 to 120	H&S-driven	
Adenosine A_1						Borea et al. 1994
Agonists	23	−60 to −25	9 to 50	205 to 356	S-driven	
Antagonists	16	−49 to −24	−44 to −12	−23 to 60	H&S-driven	
Adenosine A_{2A}						Borea et al. 1995
Agonists	7	−50 to −27	7 to 50	178 to 278	S-driven	
Antagonists	16	−50 to −26	−60 to −7	−34 to 94	H&S-driven	
Adenosine A_{2B}						Gessi et al. 2008b
Agonists	6	−43 to −29	7 to 23	123 to 219	S-driven	
Antagonists	6	−47 to 40	−40 to −20	10 to 91	H&S-driven	
Adenosine A_3						Merighi et al. 2002
Agonists	6	−54 to −41	21 to 67	225 to 410	S-driven	
Antagonists	5	−49 to −33	−52 to −9	16 to 81	H&S-driven	

Temperature used = 298.15 K; N = number of ligands; ΔG°= standard free energy; ΔH°= standard free enthalpy; ΔS°= standard free entropy; EDF = equilibrium driving force.

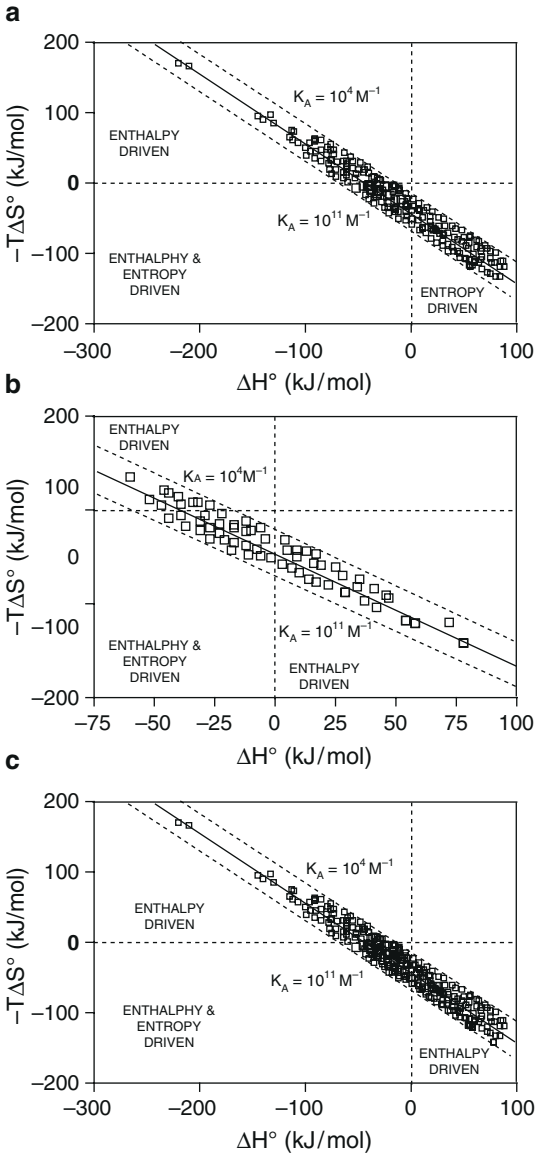

Fig. 2.3 Scatter plot of $-T\Delta S°$ versus $\Delta H°$ values for the GPCR (**a**, n = 203), LGICR (**b**, n = 68), GPCR and LGICR (**c**, n = 271) agonists and antagonists examined. All points lie on a same regression line. The two *dashed lines* indicate the loci of the points representing possible combinations of $\Delta H°$ and $-T\Delta S°$ values giving rise to the two different equilibrium constants indicated ($K_A = 10^4$ and $K_A = 10^{11}\ M^{-1}$)

receptors, the agonist binding is totally entropy-driven, ($7 \leq \Delta H^\circ \leq 50$ kJ/mol; $-83 \leq -T\Delta S^\circ \leq -53$ kJ/mol/K) and the antagonist is enthalpy- and entropy-driven ($-60 \leq \Delta H^\circ \leq -7$ kJ/mol; $-28 \leq -\Delta S^\circ \leq 10$ kJ/mol/K) (Borea et al. 1995). In a similar way, agonists at A_{2B} adenosine receptors show a totally entropy-driven binding ($7 \leq \Delta H^\circ \leq 23$ kJ/mol; $-65 \leq -T\Delta S^\circ \leq -37$ kJ/mol), while antagonist binding is enthalpy- and entropy-driven ($-20 \leq \Delta H^\circ \leq -40$ kJ/mol; $-27 \leq -T\Delta S^\circ \leq -3$ kJ/mol) (Gessi et al. 2008b). Similarly for A_3 adenosine receptors, the thermodynamic parameters fall in the ranges $21 \leq \Delta H^\circ \leq 67$ kJ/mol; $-122 \leq -T\Delta S^\circ \leq -67$ kJ/mol for agonists and $-52 \leq \Delta H^\circ \leq -9$ kJ/mol; $-24 \leq -T\Delta S^\circ \leq -5$ kJ/mol for antagonists showing that agonist binding is always totally entropy-driven while antagonist binding is enthalpy- and entropy-driven (Varani et al. 2000; Merighi et al. 2002). An overall analysis of the thermodynamic data reported in Table 2.4 indicates that the variability of ΔH° (−224 to 90 kJ/mol) and ΔS° (−590 to 456 J/mol/K) values is again much greater than that of the ΔG° values (−63 to −24 kJ/mol) suggesting the possibility that enthalpy and entropy could be proposed as indicators of the pharmacological profile of adenosine ligands. In agreement with the idea that while ΔH° values are determined by the features of the ligand–receptor binding process, ΔS° values are determined by the rearrangements occurring during the binding in the solvent–drug and solvent–receptor interfaces. As a matter of fact, in the adenosine agonist-receptor interaction the insertion of the ribose moiety and the depletion of the water network induces conformation changes in the receptor site able to mediate the final biological effect. As a consequence a high degree of correlation between intrinsic activity and ΔS° values was reported for adenosine ligands acting as full or partial agonists and as antagonists (Borea et al. 1994).

2.8 Binding Thermodynamics of Ligand-Gated Ion Channel Receptors

Analysis of thermodynamical parameters of LGICR have revealed that five out of six receptors are thermodynamically discriminated (Table 2.5, Fig. 2.3b). As for the glycine receptor, the agonist binding has to be classified as entropy-driven ($2 \leq \Delta H^\circ \leq 20$ kJ/mol; $-56 \leq -T\Delta S^\circ \leq -25$ kJ/mol), whereas the antagonist binding is mostly enthalpy-driven ($-58 \leq \Delta H^\circ \leq -15$ kJ/mol; $-15 \leq -T\Delta S^\circ \leq 29$ kJ/mol) (Gomez et al. 1989). Agonist binding to the $GABA_A$ receptor is entropy-driven ($-1 \leq \Delta H^\circ \leq 14$ kJ/mol; $-48 \leq -T\Delta S^\circ \leq -28$ kJ/mol) while antagonist binding is enthalpy- and entropy-driven ($-23 \leq \Delta H^\circ \leq -12$ kJ/mol; $-31 \leq -T\Delta S^\circ \leq -15$ kJ/mol) (Maksai 1994). A similar result is also obtained for the serotonin $5HT_3$ receptor where the agonist binding is totally entropy-driven ($18 \leq \Delta H^\circ \leq 53$ kJ/mol; $-95 \leq -T\Delta S^\circ \leq -60$ kJ/mol) and antagonist binding is both enthalpy- and entropy-driven ($-16 \leq \Delta H^\circ \leq 0$ kJ/mol; $-53 \leq -T\Delta S^\circ \leq -21$ kJ/mol) (Borea et al. 1996b). At variance with the other ion channel receptors, agonist binding to the nicotinic receptor is essentially enthalpy-driven ($-58 \leq \Delta H^\circ \leq -29$ kJ/mol; $-21 \leq -T\Delta S^\circ \leq 34$ kJ/mol)

Table 2.5 Thermodynamic parameters, ΔG°, ΔH° and ΔS° of a series of typical ligands to six ligand-gated ion channel receptors so far studied

		ΔG°	ΔH°	ΔS°		
LGICRs	N	kJ/mol	kJ/mol	J/mol/K	EDF	References
Glycine						Gomez et al. 1989
Agonists	4	−48 to −24	2 to 20	94 to 188	S-driven	
Antagonists	7	−44 to −23	−58 to −15	−45 to 97	H&S-driven	
$GABA_A$						Maksai 1994
Agonists	6	−40 to −30	−1 to 14	94 to 161	S-driven	
Antagonists	5	−48 to −30	−23 to −12	50 to 104	H&S-driven	
Serotonin $5HT_3$						Borea et al. 1996b
Agonists	7	−52 to −28	18 to 53	201 to 319	S-driven	
Antagonists	4	−53 to −37	−16 to 0	70 to 178	H&S-driven	
Nicotinic						Borea et al. 2004
Agonists	7	−51 to −25	−58 to −29	−114 to 70	H&S-driven	
Antagonists	6	−37 to -21	9 to 82	97 to 409	S-driven	
$P2X_3$ purinergic						Varani et al. 2008b, c
Agonists	5	−46 to -41	−26 to −18	59 to 73	H&S-driven	
Antagonists	6	−40 to −30	14 to 36	149 to 249	S-driven	
$P2X_1$ purinergic						Varani et al. 2008b, c
Agonists	5	−46 to −37	−31 to −23	41 to 50	ND	
Antagonists	6	−30 to −25	−22 to −19	17 to 34	ND	

Temperature used = 298.15K; N = number of ligands; ΔG°= standard free energy; ΔH°= standard free enthalpy; ΔS°= standard free entropy; EDF = equilibrium driving force.

whereas antagonist binding is totally entropy-driven ($9 \leq \Delta H° \leq 82$ kJ/mol; $-122 \leq -T\Delta S° \leq -29$ kJ/mol) (Borea et al. 1998, 2004). More recently, $P2X_1$ and $P2X_3$ purinergic receptors have been also characterised from a thermodynamic point of view with the following parameters: $-31 \leq \Delta H° \leq -19$ kJ/mol; $-15 \leq -T\Delta S° \leq -5$ kJ/mol and $-26 \leq \Delta H° \leq 36$ kJ/mol; $-74 \leq -T\Delta S° \leq -18$ kJ/mol, respectively. Interestingly, $P2X_1$ and $P2X_3$ purinergic receptors have a different thermodynamic behavior as demonstrated by the fact that agonists and antagonists for $P2X_1$ receptors show similar enthalpy and entropy values. On the contrary $P2X_3$ receptors can be considered thermodynamically discriminated because agonist binding is enthalpy- and entropy- driven and antagonist binding is totally entropy-driven (Varani et al. 2008b, c). The overall $-T\Delta S°$ versus $\Delta H°$ scatter plot of the data for GPCRs and LGICRs is reported in Fig. 2.3c.

2.9 Discussion

In the field of adenosine receptors, binding thermodynamic analysis has been performed at A_1, A_{2A}, A_{2B} and A_3 adenosine receptors and has added important findings such as the thermodynamic discrimination of agonists from antagonists and the recurrent phenomenon of E–E compensation (Borea et al. 1994, 1995, Gilli et al. 1994, Gessi et al. 2008b, Merighi et al. 2002). All the examined compounds display essentially linear van't Hoff plots indicating that $\Delta Cp°$ (standard specific heat difference of the equilibrium) values of the drug–receptor binding equilibrium is nearly zero or in other words that $\Delta H°$ values are not significantly affected by temperature in the range investigated (0–30°C). This phenomenon seems to indicate that the conformational changes needed to produce the pharmacological effect are relatively small in this class of molecules most probably because larger modifications would make the association of the receptor with the cell membrane unstable. In addition, such linearity appears to be a typical property of the drug-membrane receptor binding at variance with the most binding processes between molecules and bio-macromolecules occurring in solution (Sturtevant 1977; Tomlinson 1983; Grunwald and Steel 1995). As for all adenosine receptor subtypes it appears clearly apparent the thermodynamic interdependence of $\Delta H°$ and $-T\Delta S°$ where all the experimental points appear to be arranged along a same diagonal line, according to the equation: $\Delta H°$ (kJ/mol) = −41 (±2) + 288 (±3) $\Delta S°$ kJ/mol/K (n = 85, r = 0.981, $p < 0.001$).

For the GPCR and LGICR agonists and antagonists studied the equations were: $\Delta H°$ (kJ/mol) = −41 (±2) + 304 (±4) $\Delta S°$ kJ/mol/K (n = 203, r = 0.975, $p < 0.001$) and $\Delta H°$ (kJ/mol) = −37 (±2) + 250 (±3) $\Delta S°$ kJ/mol/K (n = 68, r = 0.965, $p < 0.001$), respectively.

The regression equation has been obtained by plotting standard enthalpy and entropy data of 271 independent experiments performed on 16 different membrane receptor systems belonging to the GPCR and LGICR families. The equation was:

$\Delta H°$ (kJ/mol) = −41 (±2) + 297 (±3) $\Delta S°$ kJ/mol/K (n = 271, r = 0.971, $p < 0.001$).

These equations could be rewritten as $\Delta H° = \beta\Delta S°$, which is the form for a case of enthalpy-entropy compensation with a compensation temperature of 302 K. It is generally accepted that entropy and enthalpy values in a scatter plot are arranged on a same diagonal band encompassed between the two dashed lines which represent the loci points defined by the limiting K_D values of 100 μM and 10 pM. This phenomenon seems to be a common feature in all cases of drug–receptor binding. The enthalpy–entropy compensation phenomenon has been attributed for drug–receptor interactions to the solvent reorganization that accompanies the receptor binding process in diluted solutions (Tomlinson 1983; Grunwald and Steel 1995). According to this point of view, while $\Delta H°$ values are most probably determined by the features of the ligand–receptor binding process, $\Delta S°$ values appear strongly affected by the rearrangements occurring in the solvent.

It seems reasonable to assume that solvent effects might be responsible for the in vitro thermodynamic discrimination between agonists and antagonists observed for the majority of LGICRs and some of the GPCRs studied (Borea et al. 2004). It is clear that for A_3 adenosine receptor agonist binding is entropy-driven but mostly enthalpy-driven for antagonists suggesting that agonists and antagonists are thermodynamically discriminated. The finding that the binding of adenosine receptor agonists is entropy-driven can be explained by the disorganization of a solvation area around to the ligand–receptor interaction. Another possible explanation is that the agonists induce a change in receptor conformation perhaps into a less-constrained state, which in turn, leads to the formation of a ternary complex with a G-protein such as ARG or AR*G and this consequently results in a decrease in the solvation of the cytosolic side of the receptor (Weiss et al. 1996). The finding of the increase in enthalpy associated with antagonist binding may be explained by hydrogen bond formation and van der Waals interactions occurring between the ligands and the binding pocket which cannot be compensated for by changes in entropy that result from agonist-induced conformational changes in the receptor. The thermodynamic discrimination is confirmed for all adenosine receptor subtypes in fact also for A_1, A_{2A} and A_{2B} adenosine receptor subtypes it has been shown that agonist binding is entropy-driven while antagonist binding is mainly enthalpy-driven (Borea et al. 1994, 1995; Gessi et al. 2008b). The entropy-driven binding of agonists has been tentatively interpreted for the A_1 adenosine receptors assuming that the ribose ring docks in a region of the binding site previously filled by water molecules which are released and responsible of the observed entropy increase. The insertion of the ribose moiety and the depletion of the water network could induce conformation changes in the receptor which are known to be associated with agonistic activity (Borea et al. 1994). The affinity and efficacy of A_1 adenosine receptor ligands are related as confirmed by the high degree of correlation between intrinsic activity and entropy values observed for a wide variety of adenosine compounds (Borea et al. 1994). In addition, the thermodynamic parameters reported for adenosine receptor subtypes suggest a very similar mechanism of ligand-receptor interaction (Gessi et al. 2008a). However, the development of therapeutic substances has been strongly advanced by rational drug design which involves achieving full understanding of a given biomolecular interaction by combining structural, kinetic and thermodynamic parameters. Therefore, the thermodynamic data represent significative and relevant informations to the drug design and development (Testa et al. 1987; Raffa and Porreca 1989; Holdgate and Ward 2005; Whitesides and Krishnamurthy 2006). In particular, when compounds have similar affinities, their enthalpy values can be used to select one as the preferred lead compound for optimization. A favourable enthalpy values implies better complementarity of the binding interfacies because enthalpy corresponds to the energy associated with the net change in non-covalent bonds. The knowledge of the thermodynamic parameters could help the discovery and characterization of novel selective A_3 agonists or antagonists.

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Chapter 3
Pharmacology and Molecular Biology of A_3 Adenosine Receptors

Karl-Norbert Klotz

Abbreviations

CCPA	2-Chloro-N^6-cyclopentyladenosine
Cl-IB-MECA	2-Chloro-N^6-3-iodobenzyladenosine-5′-*N*-methyluronamide
HEMADO	2-Hexyn-1-yl-N^6-methyladenosine
NECA	Adenosine-5′-*N*-ethyluronamide
R-PIA	R-N^6-(2-phenylisopropyl) adenosine

3.1 Introduction

Adenosine is an important metabolite and a building block for many biologically relevant molecules. Most abundantly, it contributes the purine base adenine and a ribose to ATP which as an energy-providing compound occurs in millimolar concentrations in every cell. Such high concentrations of ATP are the basis for functionally significant levels of adenosine to occur in all cells and in the extracellular space (see below). Already about 80 years ago it was discovered that adenosine exerts regulatory actions in the cardiovascular system (Drury and Szent-Györgyi 1929). It has since been appreciated that adenosine may regulate the function of almost all cells and tissues. Soon after the discovery of adenosine receptors (Sattin and Rall 1970) it became obvious that more than one subtype is responsible for the many effects that were shown to be mediated by adenosine. Initially, it was thought that two subtypes of adenosine receptors exist, A_1 and A_2 (van Calker et al. 1979; Londos et al. 1980). With the advent of routinely utilized molecular biological methods two more subtypes, A_{2B} and A_3, were found (Pierce et al. 1992; Zhou et al. 1992;

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Salvatore et al. 1993). While the existence of the A_{2B} adenosine receptor was anticipated from pharmacological data much earlier (Bruns 1980), the A_3 subtype turned out to be an entirely new adenosine receptor which was initially identified as an orphan receptor in rat testis (Meyerhof et al. 1991). Later it was cloned from various tissue and species (Zhou et al. 1992; Salvatore et al. 1993; Linden et al. 1993; Hill et al. 1997). Expression in cellular systems revealed the unique pharmacological characteristics and species differences of the A_3 adenosine receptor. For details about adenosine receptor subtypes see Fredholm et al. 2001a.

Adenosine concentrations vary widely in tissues and body fluids as it is formed both intra- and extracellularly, it undergoes metabolism by adenosine deaminase and adenosine kinase, and may be transported through the plasma membrane with equilibrative and concentrative types of transporter proteins (Zimmermann 2000; Deussen 2000; Fredholm et al. 2001a; Molina-Arcas et al. 2008). In cellular preparations the adenosine concentration in medium is found to be in the 10–100 nM range and hypoxia was shown to trigger an about twofold increase in adenosine formation (Ryzhov et al. 2007; Merighi et al. 2007a). In solid tumors even micromolar concentrations were found (Blay et al. 1997). It is not absolutely clear what level of adenosine is capable of triggering a receptor-mediated response as determination of the potency of adenosine at the four receptor subtypes is confounded by its metabolism and the presence of high amounts of endogenous adenosine even in membrane preparations. From studies by Fredholm et al. (2001b) one can conclude that 10–100 nM adenosine may be sufficient to stimulate all but A_{2B} receptors. The potency of adenosine might be higher in reality as is also suggested by binding studies with purified A_1 adenosine receptors (Freissmuth et al. 1991). Therefore, it is very likely that, for example hypoxia-mediated changes in adenosine concentrations lead to stimulation of A_3 receptors and, consequently, to relevant physiological responses (Merighi et al. 2007a, b).

3.2 Pharmacology

Like the A_1 adenosine receptor, the A_3 subtype represents an inhibitory receptor mediating a decrease in cAMP levels upon stimulation. In addition, it may also trigger a Ca^{2+} response (Kohno et al. 1996; Englert et al. 2002; Fossetta et al., 2003) or MAP kinase signaling (Schulte and Fredholm 2000; Graham et al. 2001). Agonists like CCPA or R-PIA which were previously thought to be A_1 selective show considerable affinity for A_3 receptors (Klotz et al. 1998). Nevertheless, there are numerous agonists with significant selectivity for A_3 over A_1 receptors like Cl-IB-MECA (selective in rat, Kim et al. 1994) and HEMADO (selective in human, Volpini et al. 2002; Klotz et al. 2007), thus allowing for a clear discrimination of the two subtypes employing a panel of agonists (for details see Part IV).

A plethora of antagonists selective for the human A_3 receptor was developed since its discovery. A_3 antagonists are derived from a larger number of chemical backbones than antagonists for the other subtypes (Müller 2003; Baraldi et al. 2003).

Classical adenosine receptor antagonists are xanthine and adenine derivatives; in addition, a number of compounds was developed with a triazoloquinazoline or related structures. A_3 receptors seem to tolerate a larger variety of structures, therefore, antagonists were also developed based on dihydropyridine (Jiang et al. 1997) and pyridopurinedione templates (Priego et al. 2002). 8-Substitution of adenosine which typically destroys affinity to adenosine receptors yielded also A_3 antagonists (Volpini et al. 2001) (for details see Part IV). A puzzling feature of A_3 adenosine receptors is that a large number of structurally different antagonists bind with high affinity to the human A_3 adenosine receptor while finding high affinity structures for the rat receptor remains a challenge.

3.3 Tissue Distribution

Initially after cloning, tissue distribution of mRNA coding for A_3 adenosine receptors was probed in Northern blots or with RT-PCR. The receptor transcript was found in many different tissues typically at low levels. In the case of human A_3 receptors higher levels of mRNA were found in lung and liver (Salvatore et al. 1993). Highest mRNA expression showed marked species differences with high levels in rat testis (Zhou et al. 1992) or sheep lung and spleen (Linden et al. 1993). While the A_3 message seems to occur at low level in many different brain areas of the rat (Dixon et al. 1996) a much more differentiated distribution was identified in human CNS with high expression in the medulla, spinal cord and the corpus callosum (Atkinson et al. 1997).

From functional studies important localizations were deduced including rat mast cells (Fozard et al. 1996), chicken heart (Liang and Jacobson 1998) and gerbil brain (von Lubitz et al. 1994). Radioligand binding studies revealed relevant amounts of A_3 receptor protein in colon (Gessi et al. 2004), human eosinophils (Kohno et al. 1996) and dendritic cells (Fossetta et al. 2003). The widespread appearance in cells important for inflammatory reactions and in tumor cells led to a particular interest in A_3 adenosine receptors as a target in the treatment of inflammatory diseases and cancer (see Parts IV and V). More detailed information on tissue distribution of A_3 receptors is compiled in a review by Gessi et al. 2008.

3.4 Species Differences

The A_3 adenosine receptor is different from the other three subtypes in many ways. Sequence identity of the human subtype to the human A_1, A_{2A} and A_{2B} is only 54%, 46% and 43%, respectively, compared to 56% between A_1 and A_{2A} (Table 3.1). Also, the identity between species is remarkably low compared to other receptor subtypes (Table 3.2). The considerable species variation of the A_3 adenosine receptor is also evident from a comparison of dendrograms. The high conservation of the

Table 3.1 Sequence identities (%) between human adenosine receptor subtypes. Data are taken from the 'GPCRDB All against all pairwise identity matrix' (http://www.gpcr.org/7tm_old/seq/001/001.PCTID.html)

	A_1	A_{2A}	A_{2B}	A_3
A_1	100	56	53	54
A_{2A}	56	100	65	46
A_{2B}	53	65	100	43
A_3	54	46	43	100

Table 3.2 Sequence identities (%) between adenosine receptor subtypes from different species (data are taken from the 'GPCRDB All against all pairwise identity matrix' (http://www.gpcr.org/7tm_old/seq/001/001.PCTID.html))

Human vs.	A_1	A_{2A}	A_{2B}	A_3
Rat	97	93	91	77
Mouse	97	93	93	77
Rabbit	97	–	–	78
Dog	98	95	95	91
Sheep	–	–	–	90
Guinea pig	98	–	–	–
Horse	–	94	–	–
Chick	88	–	72	–

A_1 receptor within mammals (Fig. 3.1a) contrasts the much larger differences seen for the A_3 subtype (Fig. 3.1b). In fact, the differences between human and rat A_3 are similar to the differences seen between human and the non-mammalian chick A_1 receptors. Although the pronounced sequence differences between mammalian A_3 receptors may help to explain the striking discrepancy of pharmacological characteristics between receptors in different species, it does not seem to be sufficient to account for the particularly dramatic distinction of antagonist binding to rat and human subtypes (Klotz 2000).

3.5 Gene Structure

The human A_3 receptor gene (*ADORA3*) was mapped to chromosomal location 1p13.3 (Atkinson et al. 1997). The coding sequence is found on two exons interrupted by a single intron of 2.2–2.4 kb in the region coding for the second intracellular loop (Murrison et al. 1996; Atkinson et al. 1997). This genomic arrangement seems to be identical for all four human receptor subtypes and was also found in other species (Fredholm et al. 2000). The intron–exon boundary is flanked by Arg and Tyr residues which are conserved across all receptor subtypes and species.

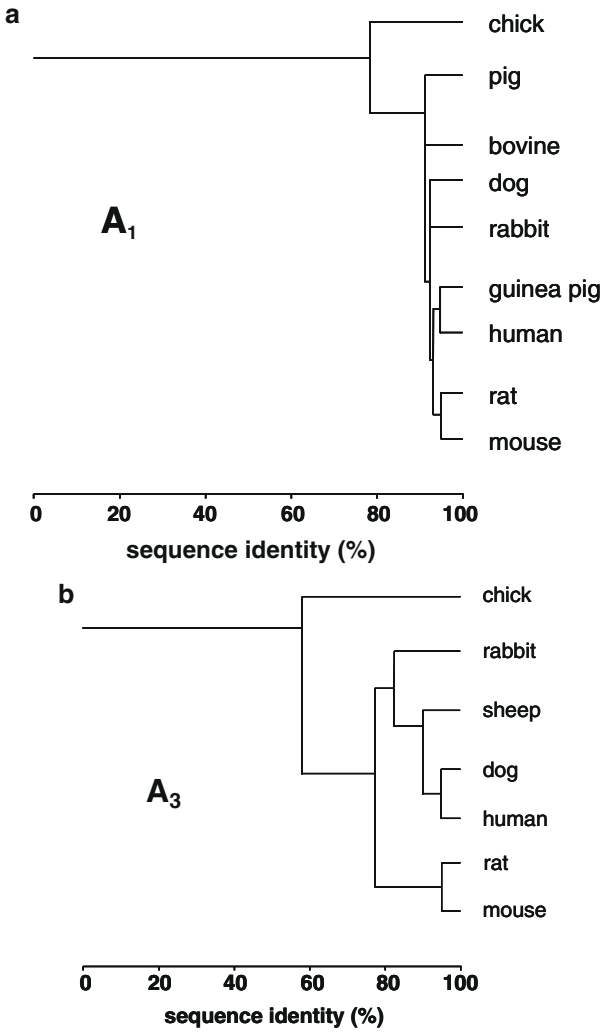

Fig. 3.1 Dendrograms of the A_1 and A_3 adenosine receptors

In the promoter region of both the human and the mouse gene no TATA or CAAT box was found at expected locations as previously found for the A_1 receptor as well (Murrison et al. 1996; Atkinson et al. 1997; Zhao et al. 1999; Fredholm et al. 2000). In accordance with results from other G protein-coupled receptor genes lacking TATA or CAAT motifs several transcription initiation sites were detected (Murrison et al. 1996; Atkinson et al. 1997; Zhao et al. 1999). In attempts to identify promoter activity in the 5′ noncoding region, gene fragments containing sequences upstream from the start codon were linked to a human growth hormone (hGH) reporter gene. Expression of a respective plasmid in various cells showed

particularly high promoter activity in a mast cell line (RBL-2H3) (Zhao et al. 1999) which is in accordance with the high mRNA expression found in mast cells (Fozard et al. 1996).

In line with expression of A_3 adenosine receptors in cells and tissues like mast cells, spleen and thymus putative binding sites for a number of transcription factors relevant for protein expression in inflammation and infection were identified in the promoter region of the A_3 receptor gene (Atkinson et al. 1997; Zhao et al. 1999). These include activator protein 1 (AP-1) which regulates gene expression in response to viral and bacterial infections or to stimulation by cytokines. Additional binding sites for transcription factors with specificity for immune cells are for the T cell-specific T cell factor F-2-α (TcF-2-α) and for the B cell-specific E2aECB.

3.6 Transgenic and Knockout Animals

Both the generation of mice overexpressing A_3 adenosine receptors and targeted deletion of the A_3 receptor gene have added important knowledge to the understanding of various functions of this adenosine receptor subtype. In particular its role in cardiovascular function was confirmed in numerous studies with genetically modified animals.

A relevant role in cardiac preconditioning was shown in mice overexpressing the A_3 receptor (Black et al. 2002). Mice carrying one or six transgene copies whose expression is under the control of the cardiomyocyte-specific promoter for the α-myosin heavy chain show reduced infarct size in a coronary occlusion model. It is important to note though that the mice with the higher expression developed all symptoms of a dilated cardiomyopathy although the lower level of overexpression did not appear to cause any functional changes to the heart. The observed protection from ischemic damage with higher A_3 receptor levels corresponds to numerous reports of cardioprotection resulting from treatment with A_3 receptor agonists (Auchampach et al. 1997; Dougherty et al. 1998).

Interestingly, targeted deletion of the A_3 adenosine receptor resulted also in a better protection from ischemic damage both in Langendorff hearts and in an *in vivo* coronary occlusion model compared to preparations from wild-type mice (Cerniway et al. 2001). It was speculated that the absence of proinflammatory A_3 effects may be responsible for the beneficial outcome of targeted deletion of the A_3 receptor. In addition, unknown compensatory changes due to the A_3 gene knockout may contribute to the paradox finding of an ischemia-tolerant phenotype (Harrison et al. 2002). A_3 knockout animals may also benefit from hemodynamic changes as adenosine stimulation results in an increased cAMP response as a consequence of a relative increase of A_2-mediated signaling (Zhao et al. 2000). Animals lacking the A_3 adenosine receptor showed, therefore, a more pronounced adenosine-induced drop in blood pressure compared to wildtype mice (Zhao et al. 2000). Along these lines, an improved coronary blood flow was observed in A_3 knockout mice (Talukder et al. 2002).

A_3 adenosine receptor knockout mice were protected from renal failure caused by ischemic-reperfusion injury or by myoglobinuria (Lee et al. 2003). A similar protection was achieved with A_3 antagonists whereas agonists worsened the condition confirming A_3 receptors as a potential target in the treatment of renal failure.

The role of A_3 adenosine receptors in modulating inflammatory responses was also confirmed in mice after its targeted deletion. Adenosine mediates an increase in cutaneous vascular permeability leading to extravasation of serum proteins as an important mechanism in the development of an inflammatory response. It turned out that this reaction is dependent on the presence of A_3 adenosine receptors on mast cells as both A_3 knockout mice and mice lacking mast cells showed no response (Tilley et al. 2000). Adenosine accomplishes mainly an anti-inflammatory effect which is generally assumed to be mediated by the A_{2A} subtype (Sitkovsky et al. 2004). However, A_3 agonists were also found to produce anti-inflammatory actions, for example by inhibiting neutrophil function. Such a contribution to inhibition of inflammation was recently confirmed in a comparison of A_{2A} and A_3 knockout mice (van der Hoeven et al. 2008).

Previous experiments with selective agonists and antagonists indicated that adenosine regulates intraocular pressure via A_3 adenosine receptors. Targeted deletion of the A_3 receptor confirmed this hypothesis (Avila et al. 2002) potentially opening up a novel therapeutic strategy to treat glaucoma, one of the leading causes of blindness.

3.7 Conclusion

The A_3 subtype is unique among the adenosine receptors in various ways. The tissue distribution triggered hope that it might be an interesting target for drug treatment in many different pathophysiological situations. Transgenic and knockout animals support this notion. It is of importance though to note that pronounced species-dependent differences in tissue distribution and pharmacology make it difficult to test potential therapeutic options in animal models. Detailed understanding of the regulation of A_3 expression in human tissues may help to develop strategies to circumvent such problems. A large selection of agonists and antagonists with high affinity for the human A_3 adenosine receptor provide a solid basis for its future as a target for adenosine-based pharmacotherapy.

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Part III
Signal Transduction

Chapter 4
Regulation of Second Messenger Systems and Intracellular Pathways

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4.1 Regulation of Second Messenger Systems Through G Protein Coupling

A_3 adenosine receptors are members of the superfamily of G-protein-coupled seven-transmembrane family of cell-surface receptors (GPCR). Although certain signals initiated by GPCRs appear not to require a contribution of one or more members of the family of heterotrimeric guanine nucleotide-binding proteins (G proteins) (Reiter and Lefkowitz 2006), GPCRs remain largely defined by their capacity to activate G proteins (Milligan and Kostenis 2006). In this activation process the activated receptor functions as a guanine nucleotide exchange factor allowing the release of GDP from a G protein α subunit and its replacement by GTP. Subsequently, either conformational rearrangements or physical separation of the GTP-bound α subunit from the β/γ subunit complex (Frank et al. 2005; Gales et al. 2006)allows both of these elements to modulate the activity of either downstream effector enzymes that synthesize secondary messengers or various ion channels (Milligan and Kostenis 2006). Second messengers, in turn, control the activity of protein kinases that regulate key enzymes involved in intermediary metabolism. The subsequent hydrolysis of the terminal phosphate of bound GTP by the GTPase activity that is intrinsic to the G protein α subunit acts to terminate these processes and functions as a kinetic restraint on signal generation (Milligan and Kostenis 2006); (Luttrell 2008).

Adenosine A_3 receptors have been shown to couple to classical or G protein-dependent second-messenger pathways through activation of both Gi family and Gq family G proteins (Palmer et al. 1995; Merighi et al. 2003; Haskó and Cronstein 2004).

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Therefore, A_3 receptor stimulation inhibits adenylyl cyclase, resulting in a reduction of intracellular cAMP levels (Zhou et al. 1992; Varani et al. 2000). Furthermore, the abilities of recombinant A_3 receptors in transfected CHO cells (hCHO-A_3) to inhibit cAMP accumulation and endogenous A_3 receptors in rat basophilic leukemia cells (RBL-2H3; a cultured mast cell line) to stimulate PLC are abolished by pretreatment with pertussis toxin (Zhou et al. 1992; Ali et al. 1990). This is consistent with a functional coupling of this receptor to G_i family G proteins. Furthermore, adenosine A_3 receptor signaling can increase phosphatidylinositol-specific phospholipase C (PLC) activity (Abbracchio et al. 1995; Ali et al. 1990; Ramkumar et al. 1993) and cause Ca^{2+} to be released from intracellular stores (Fossetta et al. 2003; Shneyvays et al. 2004, 2005; Englert et al. 2002; Gessi et al. 2001, 2002; Merighi et al. 2001). Presumably, the pertussis toxin-sensitive A_3 receptor-stimulated increase in inositol 1,4,5-triphosphate production in RBL-2H3 cells is due to increased levels of dissociated G_i-derived βγ-subunits activating phosphatidylinositol-specific phospholipase C-β isoforms, an interaction that has been demonstrated both in intact cells (Hawes et al. 1994) and with purified components (Hepler et al. 1993).

However, in experiments using the rat A_3 receptor stably expressed in a CHO cell line a functional interaction with G-proteins belonging to the $G_{q/11}$ family was demonstrated. Although derived from experiments using a heterologous expression system, this result suggests that at least in some instances the A_3 receptor-mediated activation of PLC has a pertussis toxin-insensitive element (Iredale and Hill 1993; Palmer et al. 1995). Perhaps consistent with this finding was the observation that inosine produced an increase in cytosolic calcium in hepatocytes. This effect could be blocked using an A_3 selective antagonist, but was independent of a decrease in cAMP levels (Guinzberg et al. 2006). Recently, it has been shown that the A_3 receptor signals via PLC-β2/β3 to achieve its protective effect on skeletal muscle (Zheng et al. 2007). In cardiac cells, A_3 receptor stimulation activates K_{ATP} channels, mediating A_3 receptor-dependent protection from ischemia/reperfusion injury in isolated rabbit hearts (Tracey et al. 1998). In particular, the opening of a mitochondrial K_{ATP} channel has been proposed as the end effector of preconditioning obtained through A_3 receptor stimulation in mice (Zhao and Kukreja 2002). In accordance with this hypothesis, it was recently determined that K_{ATP} channel opening is a direct effect of A_3 receptor stimulation in murine cardiomyocytes (Wan et al. 2008).

Considerable evidence supports the involvement of protein kinase C (PKC) in both the early and delayed preconditioning (Baines et al. 2001; Liu et al. 1994). PKC-δ is activated by diacylglycerol while it is unresponsive to Ca^{2+} (Kent et al. 1996). PKC-δ has been shown to have an essential role in the cellular signaling cascade that leads to the delayed protective effect of A_3 receptor stimulation in the mouse heart (Zhao and Kukreja 2003).

Furthermore, the A_3 receptor signals via the monomeric G-protein RhoA and phospholipase D to induce cardioprotection (Lee et al. 2001; Mozzicato et al. 2004).

4.2 Regulation of Intracellular Pathways

4.2.1 The A_3 Receptor and the Mitogen-Activated Protein Kinases (MAPKs) Signal Transduction Cascade

GPCRs are critical players in converting extracellular stimuli into intracellular signals. In early research on intracellular signal transduction, signaling pathways were described as linear, for example the cAMP-PKA pathway described above. Nowadays, as intracellular signaling is revealed as being an increasingly complex network, the ability of GPCRs to stimulate the regulatory pathways of the Mitogen-activated kinases (MAPKs) illustrates their influence on cell growth and differentiation. The well-conserved and diverse protein family of MAPKs consists of three main groups: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 kinases. ERKs are mainly stimulated by growth factors, while JNKs and p38 MAPK are more responsive to cellular stress and cytokines. Following this first classification, other kinases have been included into the MAPK family based on structural similarity (Miyata and Nishida 1999). MAPKs modulate the activities of various proteins including other protein kinases and transcription factors. Practically all GPCRs are capable of activating one or more MAPKs (Luttrell 2008). The adenosine A_3 receptor has been shown to be no exception (Schulte and Fredholm 2003).

There is considerable evidence for adenosine A_3 receptor-mediated effects on mitogenesis. Accordingly, the functional signalling of the adenosine A_3 receptor to MAPKs has been demonstrated in a multitude of different cellular models. The first example of A_3 receptor-mediated activation of ERK1/2 and the modulation of mitogenesis was described in human foetal astrocytes (Neary et al. 1998). This study made use of both an unselective adenosine receptor agonist (NECA) and a more selective agonist (IB-MECA) to demonstrate the selectivity of this effect towards the A_3 receptor. In addition, treatment with the inhibitor bisindolmalemide (Ro-318220) blocked this effect suggesting a role of PKC in this pathway. Subsequent and more detailed studies were performed in CHO cells stably expressing the adenosine A_3 receptor. One such study by Schulte and Fredholm demonstrated that physiological concentrations of adenosine (10–100 nM) caused a transient increase in phosphorylation of ERK1/2 that peaked after 5 min in CHO cells transfected with any one of the four adenosine receptors (Schulte and Fredholm 2000). Furthermore levels of adenosine reached during ischemia (3 μM) induce a more pronounced, but still transient, activation of ERK1/2. Thus, human A_3 adenosine receptors transfected into CHO cells are able to activate ERK1/2 at physiologically relevant concentrations of the endogenous agonist (Schulte and Fredholm 2000). It is perhaps useful at this point to highlight a potential caveat associated with some inhibitors of intracellular signalling when used to investigate the signal transduction pathways of adenosine receptors. Many such inhibitors, including genistein, chelerthrine and SQ22536 act at the ATP binding site of kinases or adenylate cyclase, respectively (Schulte and

Fredholm 2002a). Perhaps not surprisingly then, these compounds were shown to have an affinity for A_1, A_{2A} and A_3 adenosine receptors at concentrations commonly used to examine cellular signalling. However, with the judicious use of inhibitors, A_3 receptor signaling to ERK1/2 in CHO cells was shown to be dependent on βγ release from PTX-sensitive G proteins, PI3K, Ras and MEK (Schulte and Fredholm 2002b). In the same study ERK1/2 phosphorylation was shown to be independent of Ca^{2+}, PKC and c-SRC. Importantly, there are several examples of ERK1/2 phosphorylation mediated by endogenously expressed adenosine A_3 receptors. The agonist Cl-IB-MECA, by selectively stimulating the A_3 receptor in both primary mouse microglia cells and in the N13 microglia cell line, induces a biphasic phosphorylation of ERK1/2 (Hammarberg et al. 2003). In addition, functional A_3 receptors activating ERK1/2 have been also described in colon carcinoma and glioblastoma cells (Hammarberg et al. 2003; Merighi et al. 2006, 2007a). Interestingly, in the human melanoma A375 cell line it has been demonstrated that A_3 receptor stimulation was unable to activate ERK phosphorylation while the A_3 antagonists are able to improve MEKs activity (Merighi et al. 2002). Similar results were obtained in melanoma murine cells (Fishman et al. 2002). Furthermore, it has been demonstrated that stimulation of adenosine A_3 receptors inhibits A375 melanoma cell proliferation by the impairment of ERK kinase activation (Merighi et al. 2005a). Such a discrepancy may be due to the presence of different signalling pathways in different cell lines. As discussed later, in the case of the A375 melanoma cell line this result may be due to crosstalk between the PI3K/AKT pathway and the ERK1/2 pathway.

MAPKs activation has been linked to the regulation of the adenosine A_3 receptor expressed in CHO cells. This study demonstrated that inhibition of agonist-mediated MAPK activation prevented both homologous A_3 receptor desensitization and internalization by impairing phosphorylation. Furthermore, inhibition of MAPK by PD98059 prevented G protein-coupled receptor kinase (GRK2) translocation, suggesting that this kinase is a target for the A_3 receptor-mediated MAPK cascade. These results suggested that the MAPK cascade is involved in A_3 receptor regulation by a feedback mechanism that controls GRK2 activity and receptor phosphorylation. (Trincavelli et al. 2002).

Importantly, the activation of MAPKs have been implicated in ischemia/reperfusion injury. In particular it has been postulated that whereas ERK1/2 exerts a cytoprotective effect and is involved in cell proliferation, transformation and differentiation, p38 and JNK promote cell injury and death. Matot and co-workers observed an increase in phosphorylated JNK, p38, and ERK1/2 levels in lung tissue at the end of reperfusion compared with non-ischaemic control lung tissue. Interestingly, pretreatment with A_3 agonists upregulated phosphorylated ERK1/2 levels but did not modify phosphorylated JNK and p38 levels (Matot et al. 2006). This pretreatment was associated with a marked improvement in lung injury and attenuation of apoptosis after reperfusion.

Furthermore, ERK1/2 are also involved in cardiac hypertrophy and can play a protective role in ischaemic myocardium (Michel et al. 2001). Interestingly, A_3 receptor activation in rat cardiomyocytes has been demonstrated to increase

ERK1/2 phosphorylation by involving $G_{i/o}$ proteins, PKC and tyrosine kinase-dependent and -independent pathways. It has been found that Cl-IB-MECA produced a biphasic effect on cAMP accumulation with a stimulatory action starting at a concentration of 3 nM. This activity was triggered through PLC/PKC and not via direct G_s coupling (Germack and Dickenson 2004, 2005).

Besides ERK1/2, there is experimental evidence that adenosine A_3 receptors also activate p38 MAPKs in hCHO-A_3 cells (Hammarberg et al. 2004). Furthermore, it has been demonstrated that A_3 receptor stimulation is able to increase p38 phosphorylation in human hypoxic melanoma, glioblastoma and colon carcinoma cells (Merighi et al. 2005b, 2006, 2007a).

In the current literature on A_3 receptor signaling, nothing has been reported on JNK activation by A_3 receptor stimulation.

4.2.2 *The A_3 Receptor and the Phosphatidylinositol 3-Kinase/Protein Kinase B/Nuclear Factor-kB (PI3-K/AKT/NF-kB) Signal Transduction Cascade*

A_3 receptors have been associated with the PI3K/Akt pathway (Merighi et al. 2003). Active Akt causes a variety of biological effects, including suppression of apoptosis by phosphorylation and inactivation of several targets along pro-apoptotic pathways (Vivanco and Sawyers 2002). In particular, activated Akt is able to phosphorylate a variety of downstream substrates, for example the pro-apoptotic molecule Bad, caspase-9, the forkhead family transcription factors, I-K (a kinase that regulates the NF-kB transcription factor) and Raf.

There is evidence that A_3 adenosine receptor activation triggers phosphorylation of PKB/Akt, protecting rat basophilic leukemia 2H3 mast cells from apoptosis by a pathway involving the βγ subunits of G_i and PI3K-β (Gao et al. 2001). More recently, it has been demonstrated that A_3 receptors trigger increases in Akt phosphorylation in rat cardiomyocytes via a G_i/G_o-protein and tyrosine kinase-dependent pathway (Germack et al. 2004). In human melanoma A375 cells it has been shown that A_3 adenosine receptor stimulation results in PI3K-dependent phosphorylation of Akt. In particular, it has been demonstrated that serum-deprived A375 melanoma cells had no basal Akt phosphorylation whereas the A_3 receptor agonist Cl-IB-MECA treatment resulted in the phosphorylation of Akt at the Ser 573 phosphorylation site. Furthermore, it has been shown that the antiproliferative effect of Cl-IB-MECA is mediated by a PLC-PI3K-Akt signaling pathway (Merighi et al. 2005a).

Resveratrol preconditions the heart through activation of adenosine A_3 receptors protecting the heart through a cAMP response element-binding (CREB)-dependent Bcl-2 pathway in addition to an Akt-Bcl-2 pathway (Das et al. 2005a, b).

In lipopolysaccharide (LPS)-treated BV2 microglial cells A_3 receptor activation suppresses tumor necrosis factor-α (TNF-α) production by inhibiting PI3K/Akt and NF-kB activation (Lee et al. 2006). Furthermore, it has been reported that in mouse RAW 264.7 cells the A_3 receptor inhibits LPS-stimulated TNF-α release by

reducing calcium-dependent activation of NF-kB and ERK1/2 (Martin et al. 2006). According to these results, it has been demonstrated that A_3 receptor agonists exert significant anti-rheumatic effects in different autoimmune arthritis models by suppression of TNF-α production (Baharav et al. 2005). The molecular mechanism involved in the inhibitory effect of IB-MECA on adjuvant-induced arthritis included receptor down-regulation and deregulation of the PI3K-NF-kB signaling pathway (Fishman et al. 2006; Madi et al. 2007).On the contrary, it has been reported that activation of the A_3 receptor protects against ischemia/reperfusion injury in the heart through activation of NF-kB (Zhao and Kukreja 2002).

Solid tumors contain hypoxic cells that are resistant to chemotherapies such as with taxanes. Paclitaxel, the most widely studied taxane has been shown not to be highly active against newly diagnosed or recurrent glioblastoma multiforme – the most common subtype of malignant brain tumor. Interestingly, activation of PI3K-Akt-pBad (a pro-apoptotic member of the Bcl-2 family) by A_3 receptor stimulation has been recently demonstrated in human glioblastoma multiforme cells. This signaling pathway is responsible for an adenosine-mediated inhibition of paclitaxel-induced apoptosis in hypoxic conditions (Merighi et al. 2007b).

Further studies indicate that A_3 receptor activation, by interfering with PKB/Akt, can decrease interleukin-12 production in human monocytes (Hasko et al. 1998; la Sala et al. 2005).

It has been demonstrated that protein kinase A (PKA) and PKB/Akt phosphorylate and inactivate glycogen synthase kinase 3β (GSK-3β), a serine/threonine kinase acting as a key element in the Wnt signalling pathway (Fishman et al. 2002). Activation of the A_3 receptor by the agonist IB-MECA is able to decrease the levels of PKA, a downstream effector of cAMP, and of the phosphorylated form of PKB/Akt in melanoma and in hepatocellular carcinoma cells (Fishman et al. 2002; Bar-Yehuda et al. 2008). This implies the deregulation of the Wnt signalling pathway, generally active during embryogenesis and tumorigenesis to increase cell cycle progression and cell proliferation. Similar results were observed in synoviocytes from rheumatoid arthritis patients and in adjuvant-induced arthritis rats (Ochaion et al. 2008). In particular, it has been shown that a decrease in the expression levels of PKB/Akt, IκB kinase (IKK), I kappa B (IκB), NF-κB and tumor necrosis factor-alpha (TNF-α) in a rat experimental model of adjuvant-induced arthritis (AIA). In addition, the expression levels of GSK-3β, β-catenin, and poly(ADP-ribose)polymerase (PARP), known to control the level and activity of NF-κB, were down-regulated upon treatment with an A_3 receptor agonist (Ochaion et al. 2008).

4.2.3 Cross Talk Between MAPK and PI3K/AKT Signalling Pathways, and Its Modulation by the A_3 Receptor

Crosstalk between the PI3K and the Raf/MEK/ERK pathways has been reported on multiple levels, with some research stating that PI3K activity is essential for induction of Raf/MEK/ERK activity (Vivanco and Sawyers 2002; Sebolt-Leopold and

Herrera 2004). Additional studies suggest that the PI3K pathway enhances and/or synergizes with Raf/MEK/ERK signaling to provide a more robust signal through the lower components of the MAPK cascade (i.e. ERK). However, there is conflicting evidence that states that Akt is able to phosphorylate Raf, thereby efficiently abrogating Raf activity on downstream substrates (Rommel et al. 1999; Guan et al. 2000; Reusch et al. 2001; Moelling et al. 2002; Zimmermann and Moelling 1999).

In melanoma cells Akt phosphorylation mediated by the A_3 agonist Cl-IB-MECA induced Raf phosphorylation at an inhibitory phosphorylation site on Ser 259. As a consequence, Cl-IB-MECA inactivated Raf inducing a cross talk between ERK1/2 and Akt pathways in these cells (Merighi et al. 2005a). Ras-Raf-MEK-ERK pathway is normally activated by A_3 receptor stimulation as is the PI3K-Akt route. It is clear that these apparently separate routes should actually interact. A_3 receptor stimulation inhibits the proliferation of melanoma cells partly by a PLC-sensitive mechanism. Pretreatment of cells with a PLC-γ inhibitor strongly abrogated the Cl-IB-MECA effect on cell proliferation and on ERK1/2 phosphorylation, suggesting a critical role for PLC-γ in A_3 receptor signaling. Furthermore, pretreatment of A375 cells with a PI3K inhibitor and an Akt inhibitor impaired Cl-IB-MECA-induced inhibition of cell proliferation and the effects of A_3 receptor stimulation on Raf, MEK1/2 and ERK1/2 phosphorylation. These data suggest that the A_3 adenosine receptor signals through a pathway including PI3K-Akt. On the contrary, Ras was not activated. These results confirm that in A375 cells A_3 receptors decrease MEK1/2-ERK1/2 phosphorylation and cell proliferation via the inhibition of Raf, by a PI3K-Akt pathway without affecting Ras (Merighi et al. 2005a) (Fig. 4.1).

4.2.4 The A_3 Receptor and the Hypoxia-Inducible Factor 1 (HIF-1)

HIF-1 is the main transcription factor which regulates the cellular responses to hypoxia. It is a heterodimer composed of an inducibly expressed HIF-1α subunit and a constitutively expressed HIF-1β subunit. Over the last several years, HIF-1 has emerged as an attractive target for cancer therapy. Overexpression of HIF-1α protein has been reported in several human cancers, where it has been positively associated with tumor progression, treatment failure, and poor survival (Giaccia et al. 2003; Semenza 2003). HIF-1 is a potent activator of angiogenesis and invasion through its upregulation of target genes critical for these functions (Carmeliet et al. 1998; Kung et al. 2000; Ratcliffe et al. 2000). Such genes share the presence of hypoxia response elements (HRE), which contain binding sites for HIF-1 (Semenza 2003). Therefore, since HIF-1α expression and activity appear central to tumor growth and progression, HIF-1 inhibition is an attractive anticancer target (Semenza 2003). Knowledge of the mechanisms of action of all the actors in the

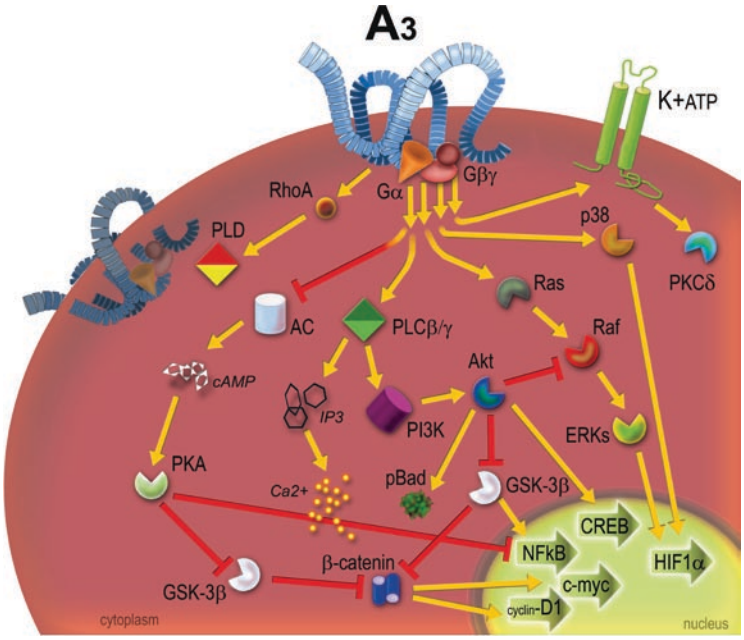

Fig. 4.1 Schematic representation of second messengers and intracellular signalling pathways mediated by A_3 receptor stimulation

hypoxic pathway is thus becoming a priority in identifying new agents capable of specifically targeting HIF-1. However, there are few choices that are currently available for direct and specific inhibition of HIF-1α. Much attention is being paid to develop new HIF-1-targeting agents. The success of these efforts will result in a new chemotherapeutic drug class which hopefully will improve the prognoses of many cancer patients. Thus far, no pharmaceutical has been identified that directly regulates the activity of a human transcription factor. Selection of the most appropriate point of therapeutic intervention to modulate HIF-1 activity is also an important factor in pharmaceutical development. In this respect, HIF-1 modulation by adenosine, increased in hypoxia (Blay et al. 1997), appears to be an attractive target for selective inhibition of the HIF-1 system in tumor hypoxic cells, without inhibition of any of the other essential HIF-1 pathways in normal cells. In particular, HIF-1 accumulation has been detected upon A_3 receptor stimulation in hypoxic melanoma, glioblastoma and colon carcinoma cells (Merighi et al. 2005b, 2006, 2007a). Furthermore, in tumor hypoxic cells, A_3 receptor activation increases vascular endothelial growth factor, VEGF, via the HIF-1 pathway revealing the functional relevance of A_3 receptor-mediated HIF-1 accumulation. The pathways involved are Akt, MEK and p38 MAPK, activated by the A_3 receptor which is able, through this signaling, to enhance HIF-1α and VEGF protein expression in tumor hypoxic cells (Merighi et al. 2005b, 2006, 2007a).

4.3 Conclusions

Over the last 20 years, the initial observation of a link between the adenosine A_3 receptor and the classical second messengers cAMP and calcium has evolved to provide evidence for new intracellular signaling pathways. The MAPK cascade and Akt-mediated signaling appear to be the principal pathways regulated by the A_3 receptor. Work of several groups has shown a modulation of the Akt/Ras/Raf/MEK/ERK signaling pathway by A_3 receptor stimulation. Of great interest are the different effects of A_3 receptor activation on the Akt/Ras/Raf/MEK/ERK signaling pathway modulation in different cells. Furthermore, there is a strong link between A_3 receptor and HIF-1 signaling in hypoxia. Although it is possible that the signaling pathways may be different in other cellular backgrounds, it seems likely that the biological events regulated by adenosine A_3 receptors under physiological and pathophysiological conditions may depend not only on changes in cAMP and Ca^{2+}, but also on mitogenic signaling via ERK1/2 and Akt. Recognition and characterization of intracellular pathways modulated by A_3 receptor advocates the principle that modulating these signaling routes is likely to constitute a considerable advance in the management of many diseases.

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Chapter 5
The Desensitisation as A_3 Adenosine Receptor Regulation: Physiopathological Implications

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5.1 A_3 Adenosine Receptor Regulatory Mechanisms

Many important physiological processes are governed by the coordinated actions of multiple receptor-mediated signalling pathways, each of which is capable of rapid and specific regulation. Achieving this regulation is highly pertinent for G protein-coupled receptors (GPCRs) which represent the largest family of signalling receptors expressed in animals which respond to a wide range of stimuli. These regulatory mechanisms, which may be activated by receptor agonist occupancy or occur in a agonist independent manner, include receptor "desensitisation", "internalisation" and "down-regulation", all contributing to the interruption of receptor responsiveness over time.

The A_3 adenosine receptor (A_3 AR) is the last adenosine receptor to be cloned, and it is the only adenosine subtype that was cloned before its pharmacological identification. Recently, Gessi et al. (2008) have defined A_3 AR as "an enigmatic player in cell biology" in their review describing how the A_3 AR activation can play multiple roles in different therapeutic fields. The regulation of A_3 AR (for a review, see Klaasse et al. 2008) is very important because it is involved in a variety of significant physiological processes, including modulation of cerebral and cardiac ischaemic damage (Von Lubitz et al., 1999; Liang and Jacobson 1998), inflammation (Akkari et al. 2006), modulation of intraocular pressure (Mitchell et al. 1999), regulation of normal and tumour cell growth (Brambilla et al. 2000; Baraldi et al. 2005; Gessi et al. 2004) and immunosuppression (Fredholm et al. 2000; Fishman and Bar-Yehuda 2003).

Exposure of any GPCR to its agonist for shorter or longer times generally leads to an attenuation of the agonist response (Krupnick and Benovic 1998; Lefkowitz 1998; Pitcher et al. 1998; Ferguson 2001). Adenosine receptor are no exception (Olah and Stiles 2000); however, the magnitude of the response and the mechanism involved seem to be different for the four adenosine subtypes.

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Agonist-induced desensitisation can be divided into two temporally and mechanistically distinct phases: (a) a short-term agonist exposure can induce receptor phosphorylation, preventing receptor/G-protein interaction (Hausdorff et al. 1990; Ramkumar and Stiles 1994); (b) a long-term agonist treatment can result in down-regulation of the receptor and/or of its associated G-protein, as well as the up-regulation of components controlling opposing signalling pathways (Ramkumar and Stiles 1994). For GPCR desensitisation mechanisms, see Box 5.1.

Box 5.1 GPCR Desensitisation

The desensitisation of a GPCR response can be described as the loss of response subsequent to prolonged or repeated administration of agonist (Hausdorff et al. 1990). Actually the term *prolonged* can be somewhat misleading as experimentally this can represent time periods of as little as a few seconds or as long as several hours or even days. Desensitisation can be homologous or heterologous in nature: homologous desensitisation refers to the loss of response solely to agonists that act at a particular GPCR subtype (Kelly et al. 2008), whereas heterologous desensitisation refers to a more generalised effect involving the simultaneous loss of agonist responsiveness at multiple GPCR subtypes even in the absence of specific receptor agonist occupation. Homologous desensitisation is usually thought to involve adaptative changes at the level of the GPCR itself (see Fig. 5.1), whereas heterologous desensitisation may also involve changes in signalling components downstream of the GPCR (see Fig. 5.2).

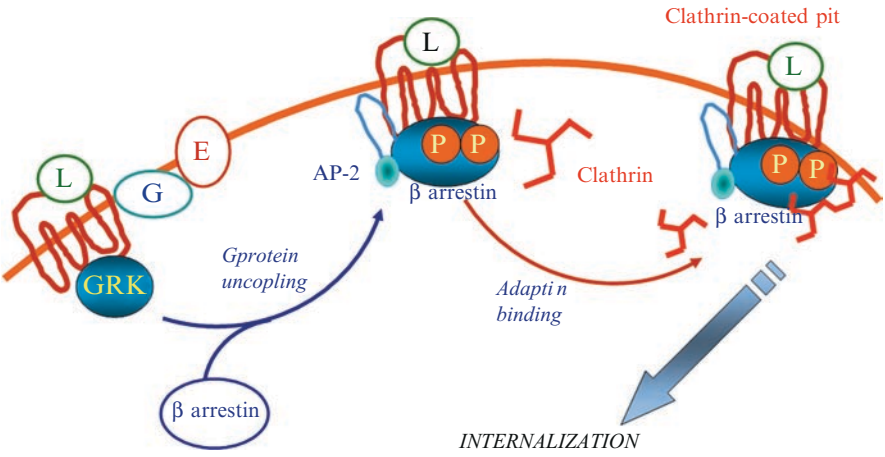

Fig. 5.1 G protein-coupled receptor (GPCR) regulation trough homologous desensitisation. The classical model of GPCR regulation by GRKs and arrestins. The agonist (L) occupied GPCR is phosphorylated by GRK; arrestins binds to the phosphorylated receptor leading to G protein uncoupling, desensitisation. The binding of arrestin to the receptors also induced the association of the receptor with other accessory proteins (adaptin) promoting receptor internalisation. G: G protein; E: effector

Box 5.1 (continued)

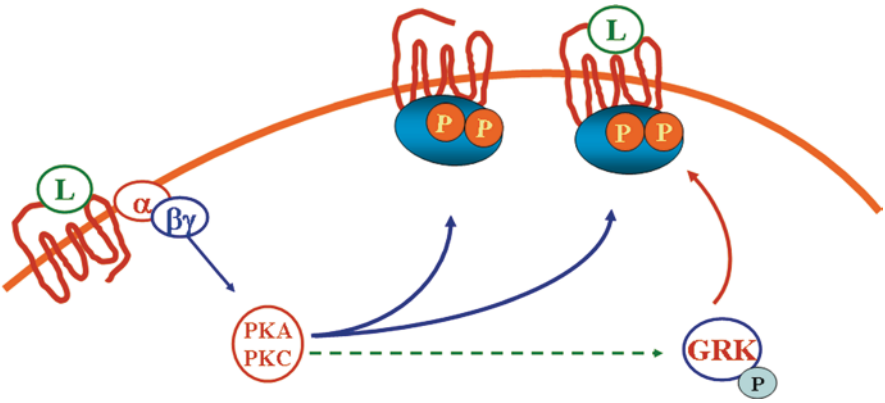

Fig. 5.2 G protein-coupled receptor (GPCR) regulation trough heterologous desensitisation. Agonist binds to the GPCR leading to second messenger activation. These kinases are then able to phosphorylate both agonist occupied and unoccupied receptors preventing GPCR coupling to G proteins. PKC can also phosphorylate and activate GRK2, which consequently has an enhanced ability to phosphorylate the GPCR

A major mechanism underlying desensitisation is the phosphorylation of the GPCR (Stadel et al. 1983; Krupnick and Benovic 1998; Pitcher et al. 1998; Ferguson 2001; Tobin 2008; Tobin et al. 2008) induced by protein kinases that mediate multi-site phosphorylations (Lohse 1993; Lefkowitz 2004) and that play a key role in determining the signalling properties of this receptor superfamily. The most important protein kinases involved in GPCR regulatory mechanisms are the G protein-coupled receptor kinases (GRKs) and the second-messenger protein kinases, including protein kinase A (PKA) and protein kinase C (PKC). The former are involved in homologous desensitisation (Freedman and Lefkowitz 1996; Premont and Gainetdinov 2007), whereas the latter mediate heterologous regulation of GPCRs. These different protein kinases have different mechanism of action: they phosphorylate distinct sites on the receptor with different kinetics, and the signalling consequence of these phosphorylation events have some overlapping features (e.g. they both result in receptor desensitisation) as well as unique features (e.g. the ability to induce receptor internalisation).

The widely accepted model of GPCR homologous desensitisation describes a system in which the agonist-activated receptors couple to G proteins to induce a cellular response, and are subsequently phosphorylated by members of GRK, with GRKs invariably phosphorylating serine or threonine residues on the third intracellular loop or COOH terminus of the receptors (Reiter and Lefkowitz 2006). The GRK family consists of seven different genes.

(continued)

Box 5.1 (continued)

The GRKs have been divided into three protein subfamilies, based on sequence similarity: GRK_1 and GRK_7 belong together; the second subfamily consists of GRK_2 and GRK_3 (the major GRK responsible for agonist-dependent desensitisation); GRK_4 and GRK_5 and GRK_6 form the third subfamily and are constitutively associated with the membrane.

The primary function of GRK-mediated receptor phosphorylation is to promote the recruitment of another crucial protein, named β-arrestin,to the activated receptor. Arrestin binds to GPCRs and inhibits further coupling to G proteins hence desensitizing the response (Ferguson 2001; Penn et al. 2001; Pierce et al. 2002; Maudsley et al. 2005). β-arrestin belongs to the arrestin family of which four members have been identified (Gurevich et al. 2008).

In addition to their role in desensitizing the GPCR response, arrestins are also central to GPCR trafficking interacting with specific proteins involved in receptor internalisation machinery (Reiter and Lefkowitz 2006; see below). Recent observations have added complexity to the classical model of GPCR desensitisation. First it is clear that, apart from mediating desensitisation, GRK and arrestins are also able to act as signal initiators by acting as multi-protein scaffolds, leading for example to arrestin-dependent activation of mitogen-activated protein kinases (DeWire et al. 2007; Ribas et al. 2007). In summary, GRKs and arrestin orchestrate GPCR activities at three different levels: (1) silencing which is the functional uncoupling of the receptor from its G protein by a mechanism known as "homologous desensitisation", (2) trafficking, which involves receptor internalisation, resensitization and/or degradation, and (3) cross-signalling which involves activation or inhibition of intracellular signalling pathways, independent of heteromeric G proteins (Reiter and Lefkowitz 2006).

Following the discovery of the role of GRK and arrestins in GPCR desensitisation, second messenger-dependent protein kinases seemed at times to be relegated to a secondary role. However, their importance in GPCR regulation has been reassessed in the light of more recent studies. For example, it is clear that the desensitisation of specific purinergic receptors (i.e. $P2Y_1$) is mediated almost exclusively by feed-back phosphorylation induced by these kinases with GRK playing only a marginal role (Hardy et al. 2005). It is unclear exactly how GPCR phosphorylation by second messenger-dependent protein kinases uncouples the receptor from its G protein, but phosphorylation of the receptor in a G protein-coupling region may sterically inhibit interaction with the G protein (Benovic et al. 1985). These kinases are able to phosphorylate agonist-unoccupied GPCRs, indicating that this type of phosphorylation could underlie some forms of heterologous desensitisation (Clark et al. 1988) In some cases, both second messenger-dependent protein kinases and GRKs

(continued)

Box 5.1 (continued)

are able to phosphorylate and desensitize the same GPCR (Hausdorff et al. 1990; Castro et al. 2002; Ally et al. 2003). Such a combination of phosphorylation events by different kinases can lead to additive effects on desensitisation. A further action of second messenger-dependent protein kinases concerns the modulation of GRK function at GPCRs: it has been demonstrated that PKC could phosphorylate and activate GRK_2 (Chuang et al. 1995; Krasel et al. 2001), with the PKC phosphorylation enhancing the ability of GRK_2 to target the plasma membrane and hence phosphorylate agonist-occupied receptors. Finally, these kinases could phosphorylate other proteins, that subsequently influence GPCR desensitisation, such as regulator of G protein signalling proteins and others (Cunningham et al. 2001; Lorenz et al. 2003).

Whereas the majority of receptor phospho-acceptor sites are in serine-and-threonine-rich regions of the intracellular domains, there is also evidence that GPCRs are phosphorylated by different kinases (CK_2, CK_1, Akt, PKB) on tyrosine residues (Paxton et al. 1994; Fan et al. 2001) in a manner that can, in some instances, generate classical phosphotyrosine protein-interaction motifs (Karoor et al. 1998).

The first studies about A_3 AR desensitisation were conducted in CHO cells stably expressing rat A_3 AR. In these cells, a short-term agonist exposure (about 10 min) results in a 30–40% reduction in A_3 receptor cell expression. This reduction is associated with a functional desensitisation of A_3 ARs as shown by an eightfold increase in the IC_{50} value for IB-MECA mediated inhibition of forskolin-stimulated adenylyl cyclase activity (Palmer et al. 1995, 1996). In this cell line, the rat A_3 AR internalises rapidly after NECA or R-PIA treatment in about 10 min (Ferguson et al. 2000). A similar rapid desensitisation has been observed in a rat basophilic leukaemia cell line, RBL-2H3 which natively expresses the A_3 AR: in this case the agonist NECA induces a transient increase of intracellular Ca^{2+} levels. Interestingly, a second challenge with NECA leads to a partial desensitisation of the initial response (Ramkumar et al. 1993).

CHO cells have been also used to study the human A_3 AR desensitisation mechanisms, in order to evaluate some discrepancy in human and rat A_3 AR regulation due to the structural and pharmacological differences between the two species homologues.

In CHO cells, stably expressing human A_3 AR, a prolonged treatment with the NECA agonist (20 h), induces uncoupling of the receptor from Gi-protein and functional desensitisation associated with a reduction in the number of the high affinity binding sites, in accordance with the data about the rat A_3 AR.

Despite this A_3 AR desensitisation, the adenylyl cyclase activity is not reduced, but almost a twofold increase is detected in the presence or in the absence of forskolin. This effect seems not to be mediated by e new receptor synthesis, but is probably due to an increased coupling efficiency between Gs and adenylyl cyclase. The sensitization of adenylyl cyclase activity is evidence that acute and chronic treatment of adenosine receptors might lead to opposite effects (Palmer et al. 1997).

The agonist-induced internalisation and the relationship between internalisation and signal desensitisation and re-sensitization were also studied in CHO cells stably expressing the human (h)A_3 AR. IAB-MECA agonist exposure induces hA_3 internalisation with a half-life of 17 min and by immunogold electron microscopy hA_3 was localized in intracellular vesicles. Subsequent removal of the agonist leads to restoration of receptor functioning and recycling to plasma membrane in about 35 min (Fig. 5.3). In this study Trincavelli and co-workers (2000) also showed that the hA_3 AR internalisation occurs via clathrin-coated pits as demonstrated by the using of the inhibitor of these endocytic pathways (hypertonic sucrose, concanavalin A and potassium depletion). Short-term exposure to agonist causes a rapid desensitisation as determined by an adenylyl cyclase assay. Removal of the agonist leads to a re-sensitization of cAMP signal to 90% of the control values within 120 min (Trincavelli et al. 2000). The desensitisation, internalisation and down-regulation has also been also investigated in human astrocytoma cells natively expressing hA_3 AR. Short-term exposure to a selective A_3 agonist, Cl-IBMECA, causes a rapid desensitisation of the receptor within 15 min, accompanied by rapid internalisation

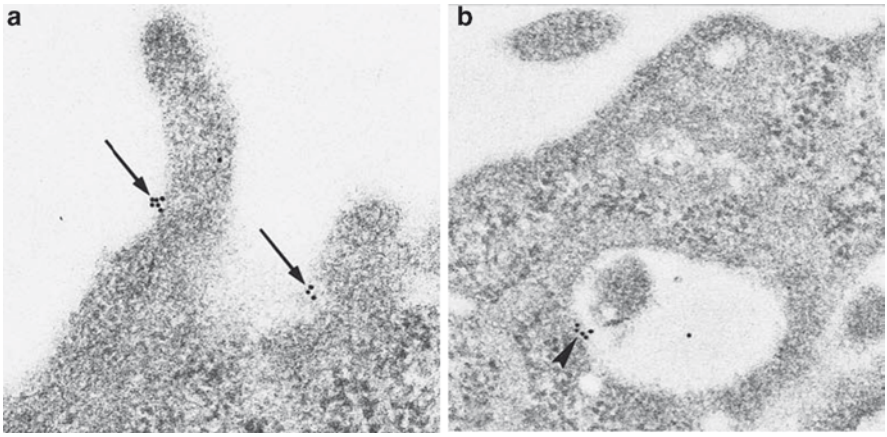

Fig. 5.3 Immunogold labeling of human A_3 ARs on ultrathin sections of transfected CHO cells. (**a**) Small aggregates of gold particles (*arrow*) on the plasma membrane after a 60-min incubation with 10 nM agonist NECA at 4°C. (**b**) After a 15-min incubation with agonist at 37°C A_3 ARs are visible in uncoated vesicle at level of the cortical cytoplasm (Trincavelli et al. 2000)

with a rapid kinetics (30 min). After 10 min A_3 AR has been found in smooth-surface pits and in uncoated vesicles in the cytoplasm. After 30 min the receptor is found in vesicular endosomes. After desensitisation, the removal of the agonist leads to the recovery of receptor functioning through receptor recycling to cellular surface within 120 min. Prolonged agonist exposure (1–24 h) results in a marked down-regulation (about 22% of control values), and the restoration of receptor function associated with receptor levels close to control values is very slow (24 h) (Trincavelli et al. 2002a).

Moreover, to investigate the species differences between rodent and human homologues, A_3 AR-humanised mice (A_3 $AR^{h/h}$) were generated. In these mice, the mouse A_3 AR gene was replaced but its human counterpart. The expression level of the human A_3 AR in A_3 $AR^{h/h}$ mice is equivalent to that of the mouse A_3 AR in wild-type mice. The agonist-mediated human A_3 AR elevates the intracellular [Ca^{2+}] in bone marrow-derived mast cells (BMMCs) derived from A_3 ARh/h mice; this elevation is completely antagonised by a highly potent and selective antagonist for the human A_3 AR, KF26777. However, the agonist-stimulated human A_3 AR is unexpectedly unable to elicit the improvement of IgE/antigen-dependent mast cell degranulation in the humanised BMMCs. Moreover, the rate of human A_3 AR internalisation, which is known to be caused by MAPK activation (Trincavelli et al. 2002b), in the BMMCs is lower than that observed in the BMMCs from wild-type mice. The reason why the human A_3 AR is slightly internalised by the A_3 AR agonist despite no phosphorylation of ERK1/2 remains to be solved (Yamano et al. 2005).

The A_3 AR trafficking and functioning has been associated with the inhibition of tumour growth in murine B16-F10 melanoma cells. In fact, the A_3 AR activation is involved in melanoma cell growth by deregulating protein kinase A and key components of the Wnt pathway, which is involved in melanoma proliferation (Robbins et al. 1996; Morin 1999; Bonvini et al. 2000). Exposure to 10 nM IB-MECA (5 min) causes a rapid internalisation to the endosomes for a recycling, whereas a long exposure (60 min) leads to a receptor localisation into lysosomes for degradation. Receptor distribution in the lysosomes is consistent with the receptor down-regulation and is followed by mRNA and receptor re-synthesis. At each stage, the modulation of cAMP production and the downstream effectors PKA, glycogen synthase–3β, c-Myc and cyclin D1 (involved in Wnt pathway) has also been evaluated to asses the receptor functionality. In particular, a decrease in PKA and an increase in GSK–3β levels have been detected. These data confirm previous observations of a cross-talk between A_3 AR and Wnt pathways in the inhibition of melanoma cell growth. The employment of 100 nM MRS 1523, an A_3 AR antagonist, counteracts the receptor internalisation as well as the modulation of the Wnt pathway, demonstrating that the responses are A_3 AR-mediated. In an animal model of melanoma tumour, the administration of IB-MECA significantly inhibits tumour growth at the same time a decrease in A_3 AR expression occurs in tumour lesions, in accordance to the in vitro experiments conducted in the B6-F10 cell line (Madi et al. 2003) (for GPCR internalisation mechanisms see Box 5.2).

Box 5.2 GPCR Internalisation

Receptor desensitisation, initiated by phosphorylation of the receptor, can be subsequently followed by receptor internalisation via multiple methods including clathrin-coated pits and/or lipid rafts/caveolae. Clathrin-coated pits are specialized regions of the cell surface that mediates the internalisation of the most of GPCRs to endosomes. Lipid rafts are planar domains in cell membranes that are enriched in specific lipid and proteins with an high content of cholesterol and glycosphingolipid (Chini and Parenti 2004). Caveolae are flask-shaped invaginations located at or near the plasma membrane and are considered a non-planar subfamily of lipid rafts (Ferguson 2001; Chini and Parenti 2004).

The internalisation pathways for GPCRs are specific for each receptor subtype, cell type, cell metabolic state, and other cell specific factors etc. Receptor trafficking may be regulated in different ways: (1) the receptor resides mainly in lipid rafts/caveolae and enters the cell via this pathway by default; (2) the receptor leaves lipid rafts upon agonist binding to be internalised via clathrin-coated pits; (3) the receptor moves into lipid rafts upon agonist binding and is internalised by this way; (4) the receptor moves into lipid rafts after agonist binding and activates certain signalling events, but is eventually moved out of the lipid rafts to be internalised via the chlatrin pathway. Among these pathway, the endocytosis via clathrin-coated pits remains the most important pathway of GPCRs trafficking. Upon phosphorylation, β-arrestin not only interacts with the receptor preventing receptor-G protein interaction but also binds to specific proteins of the endocytic machinery, including the heavy chain of clathrin, the b2 adaptin subunit of the clathrin adaptor protein AP2 and to phosphoinositides, promoting receptor internalisation via clathrin-coated pits (see Fig. 5.4). These interactions direct the phosphorylated receptor to punctuate clathrin-coated pits in the cell membrane, which are internalised by activation of the GTPase dynamin. Upon internalisation, receptors can either be rapidly recycled to the plasma membrane, targeted to larger endosomes and slowly recycled, or degraded in lysosomes. The final destination of the internalised receptors largely depends on the B-arrestin subtype that is recruited by the receptor upon phosphorylation and on the duration of arrestin binding (Hanyaloglu and von Zastrow 2008).

The cellular fate of a receptor can be determined at multiple points in the endocytic pathway and via receptor association with many different cytosolic proteins (Marchese et al. 2008). Sorting the internalised receptors to lysosomes promotes proteolytic degradation of the receptors, preventing receptors from signalling again and producing a prolonged attenuation of cellular signalling. Receptor down-regulation in lysosomes is solely a consequence of to receptor internalisation inside the cell; thus, although downregulation of a GPCR invariably adds to the overall desensitisation of a GPCR response,

(continued)

Box 5.2 (continued)

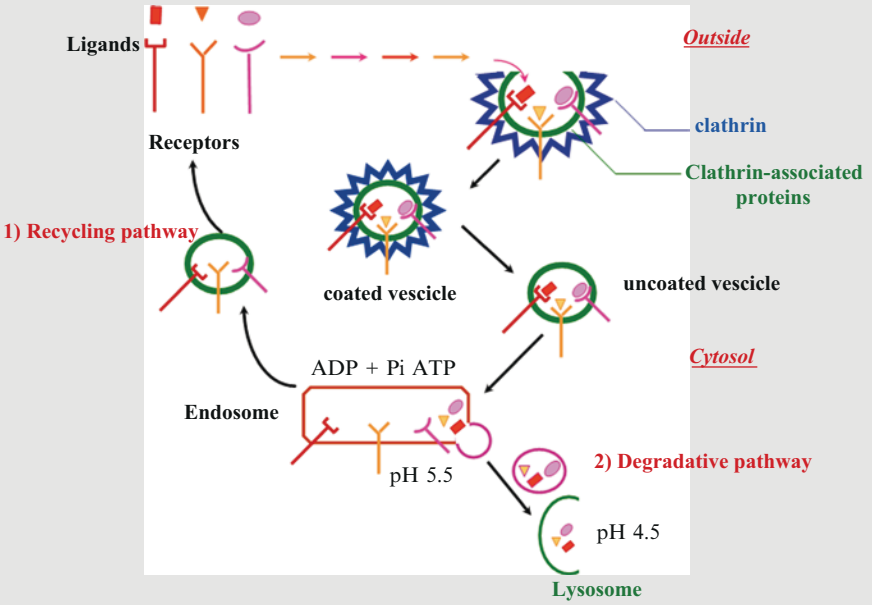

Fig. 5.4 Endocytosis of GPCRs mediated by GRKs, arrestins and clathrin-coated pits. Receptor phosphorylation mediates recruitment of arrestins from the cytoplasm, the arrestin interaction with other adaptotor proteins, and promotes receptor endocytosis by clathrin-coated pits. Internalised receptors may be sorted into a rapid recycling pathway, or to a degradative pathway into the lysosomes

most GPCRs can undergo extensive desensitisation (particularly following acute agonist addition) without any down regulation being detectable.

Sorting of internalised receptors into a rapid recycling pathway, by contrast, promotes the return of intact receptors to the plasma membranes and effectively resensitizes cells to respond again to extracellular ligand (Hanyaloglu and von Zastrow 2008, see Fig. 5.4). Repeated rounds of ligand-induced endocytosis can effectively re-route "recycling" receptors to the lysosomal pathway, resulting in an altered endocytic itinerary that contributes to down regulation of receptors often produced under conditions of chronic agonist exposure. GPCR recycling is a targeted and highly specific process, at least in mammalian cells, requiring specific cytoplasmic sequences for efficient return of endocytosed receptors to plasma membranes. These recycling sequences are highly diverse and interact specifically with distinct cytoplasmic sorting proteins, suggesting a combinatorial mechanism controlling the endocytic regulatory profile of individual GPCRs in complex mammalian cells.

5.2 Molecular Mechanisms

Rapid termination of signalling by GPCRs is typically initiated by receptor phosphorylation events which are catalysed by second messenger activated-kinases or by GRKs (Lohse 1993).

The role of receptor phosphorylation in regulating A_3 AR signalling was evaluated in a CHO cell line stably expressing rat A_3 AR. The exposure of these cells to 10 μM NECA for 10 min induces rat A_3 AR phosphorylation. Phosphoamino acid analysis reveals that phosphorylation is predominantly on threonine residues with some phosphoserine also being detected, but no phosphotyrosine (Palmer et al. 1995). The ability of NECA to stimulate rat A_3 AR phosphorylation is dose dependent and extremely rapid: in fact, the phosphorylation is already detectable after 15 s, is maximal by 4 min, and is sustained for 20 min in the presence of agonist. In order to evaluate which kinases are involved in this mechanism, several second messenger regulated-kinases, such as PKC, Ca^{2+}-calmodulin kinases, and cyclic nucleotide-dependent kinases, were activated, and all failed to stimulate A_3 AR phosphorylation under conditions in which NECA is effective. The lack of any effect of such kinases strongly suggests the involvement of one or more GRK isoforms in A_3 AR phosphorylation because these kinases specifically phosphorylate agonist-occupied receptors (Lohse 1993; Premont et al. 1995). In vitro experiments show that GRK_2 enhances the agonist dependent rat A_3 AR phosphorylation observed in isolated membranes. Interestingly, the pre-treatment of transfected cells with agonist reduces the subsequent level of GRK_2-stimulated agonist dependent A_3 AR phosphorylation observed in vitro. One possible explanation of these phenomena would be that agonist pre-treatment induces A_3 AR phosphorylation in situ on some of these residues by GRK_2 such that they are not available for subsequent phosphorylation in vitro (Palmer et al. 1995). The generation of a chimeric hA_1-rA_3 AR demonstrated that the structural determinants conferring rapid A_3 AR desensitisation kinetics and sensitivity to GRK-mediated phosphorylation are located within a small region of 14 amino acid at its C terminus (Palmer et al. 1996). The simultaneous mutation of Thr^{307}, Thr^{318}, and Thr^{319} to Ala residues in the C terminal domain dramatically reduces agonist stimulation phosphorylation and rapid desensitisation of rat A_3 AR. Individual mutation of such residues demonstrates that Thr^{318} and Thr^{319} are the most important sites for phosphorylation. In addition, phosphorylation of Thr^{318} seems to be necessary to observe phosphorylation at Thr^{319}, but not vice versa, showing that the phosphorylation of Thr amino acids in the C-terminal domain proceeds in an ordered fashion. Moreover, the replacement of Thr^{318} with a negatively charged residue (Glu) is insufficient to rescue phosphorylation at Thr^{319}, suggesting that additional complex conformation changes associated with rat A_3 AR phosphorylation may be required. In addition, the mutation of two predicted palmitoylation-sites, $Cys^{302,305}$, proximal to the regulatory domain, results in agonist-independent basal phosphorylation of the rat A_3 AR. Such findings indicate that these cysteine residues play an important role in controlling the accessibility of A_3 AR C-terminal regulatory domain to activated GRKs (Palmer and Stiles 2000).

Studies performed on human A_3 AR stably transfected in CHO cells show the involvement of extracellular regulated kinase (ERK 1/2), members of the MAPK

family, in A_3 AR phosphorylation. In particular, A_3 AR mediates the activation of ERK ½ with typical transient monophasic kinetics (5 min). The exposure of the cells to PD98059, a MAPK kinase inhibitor, prevents MAPK activation and inhibits homologous A_3 AR desensitisation and internalisation, impairing agonist-mediated receptor phosphorylation. PD98059 also inhibits the membrane translocation of GRK_2, suggesting that these kinases are a target for the MAPK cascade. These findings indicate that the MAPK pathway is involved in A_3 AR regulation by a feedback mechanism, which controls GRK_2 activity and probably involves a direct receptor phosphorylation (Trincavelli et al. 2002b).

The activation of rat A_3 AR causes the accumulation of arrestin3, both at plasma membranes and also within discrete punctate intracellular spots. This translocation seems to be correlated with receptor sensitivity to GRK-mediated phosphorylation, as demonstrated by the generation of a chimeric hA_1-r A_3AR. In contrast to other GPCRs, which display significant co-localization with the arrestin3 following agonist stimulation, the A_3 AR directed arrestin3 trafficking into punctate clusters, which are devoid of receptor immunoreactivity (Ferguson et al. 2002). In RBL-2H3 cells, which highly express rat A_3 AR, neither recruitments of arrestin3 and arrestin2 was not detected after NECA stimulation, and also no changes in A_3 AR distribution were observed. Two explanations are possible for these phenomena: the first is that A_3 AR follows an endocytic mechanism that does not involve arrestin-mediated clathrin-coated pits internalisation; the second is that the arrestin recruitment is below the limit of detection in the RBL-2H3 cells (Santini et al. 2000).

5.3 Physiopathological Implications

GPCR regulatory mechanisms, controlling receptor responsiveness over time, may play an important role in both physiological and pathological conditions and may represents a useful target against which to develop new therapeutic strategies. In addition, there is now a gathering body of evidence that indicates that GPCR phosphorylation, and then desensitisation processes, involve a range of different protein kinases able to phophorylate the same receptor at different sites and that this results in differential signalling outcomes (Tobin et al. 2008). In this regard, GPCR regulation represents a flexible regulatory process where the recruitment of different protein kinases in cell types could tailor the signalling response of the receptor to suit a particular physiological role. If this mechanism is true, then one would expect to see different patterns of receptor regulation in different cell types. This may prove to be a very important point in terms of drug discovery and design. In fact, since GPCRs act in a tissue-specific manner, targeting regulatory receptor processes in each tissue and targeting these in therapeutic strategies may limit the side effects common to many GPCR drugs.

Adenosine receptors are widespread throughout the body and exert many different functions both in the central nervous system (CNS) and in the periphery. The A_3 AR is expressed in CNS at low levels, mainly in hypothalamus and thalamus (Zhao et al. 2002). The highest levels of these receptors have been found in the lung and liver, in eosinophils, mast cells, kidney, heart spleen and other peripheral tissues (Fredholm et al. 2001; Young et al. 2004; Yaar et al. 2005). The A_3 AR has been

implicated in mediating allergic responses, airway inflammation, and apoptotic events, although the latter is dependent on the cell type and/or the type of activation (Fredholm et al. 2001; Young et al. 2004). Furthermore, A_3 AR is involved in the control of the cell cycle and inhibition of tumour growth (Yaar et al. 2005). In fact, adenosine A_3 ARs have been demonstrated to be more highly expressed in tumours than in healthy cells, suggesting a role as a tumour marker. Finally, in the CNS, A_3 ARs have been implicated in the modulation of ischaemic cerebral damage, with neuroprotective and neurodegenerative effects dependnt upon ligand concentrations and time exposure (Jacobson et al. 1999). Whereas nanomolar agonist concentrations induce cytoprotective effects (Abbracchio et al. 2001), micromolar concentrations impair cell cycle progression causing cell death (Abbracchio and Burnstock 1998). In addition opposite effects may be obtained following acute or chronic A_3 AR stimulation. It has been demonstrated that the chronic receptor activation is cerebroprotective against ischaemia damage (von Lubitz et al. 1999). On the contrary, enhanced mortality and extensive neuronal destruction is induced by acute agonist administration (von Lubitz et al. 1994). Such regimen-dependent inversion of agonist-dependent effects (in dependence of both time and concentrations) may be related to adaptive changes in A_3 AR induced by receptor desensitisation and internalisation. Moreover, the different A_3 effects that are detected in specific tissues in different species, may be ascribed to specific regulatory mechanism involving different protein kinases expressed in each tissue. As A_3 AR undergoes to rapid desensitisation, chronic administration of low agonist concentrations, in addition to antagonists, may be used as neuroprotective agents.

The same regulatory mechanisms may be important to develop A_3 agents in therapy of tumour progression. Madi et al. (2003) have examined the association between A_3 AR trafficking and receptor functionality and tumour growth inhibition induced by A_3 agonists in melanoma cells. Cell exposure to low agonist concentrations induced a block in tumour cell proliferation that parallels with A_3receptor internalisation and down-regulation. These results support the important role of A_3 AR regulation in A_3-mediated effects in pathological conditions.

In conclusion, the understanding the complexity of A_3 AR regulatory mechanisms in each tissue may reveal important targets for the development of specific therapeutic strategies in different pathological conditions, eliminating several collateral side effects observed when non-tissue-specific therapeutics are used.

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Part IV
Medicinal Chemistry

Chapter 6
A_3 Adenosine Receptor Agonists: History and Future Perspectives

Kenneth A. Jacobson, Zhan-Guo Gao, Dilip K. Tosh, Gangadhar J. Sanjayan, and Sonia de Castro

Abbreviations

AR	Adenosine receptor
BAY-60-6583	2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide
cAMP	Adenosine 3′,5′-cyclic phosphate
CHO	Chinese hamster ovary
Cl-IB-MECA	2-Chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine
CP608039	(2S,3S,4R,5R)-3-amino-5{6-[5-chloro-2-(3-methylisoxazol-5-ylmethoxy)benzylamino]purin-9-yl-l-4-hydroxytetrahydrofuran-2-carboxylic acid methylamide
DBXRM	7-β-D-Ribofuronamide
DU124183	2-Cyclopentyl-4-phenylamino-1H-imidazo[4,5-*c*]quinoline
DU124183	*N*-phenyl-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine
GPCR	G protein-coupled receptor
h	Human
HEK293 cells	Human embryonic kidney 293 cells
I-AB-MECA	N^6-(4-Amino-3-iodobenzyl)-5′-*N*-methylcabroxamidoadenosine
IB-MECA	N^6-(3-Iodobenzyl)-5′-*N*-methylcarboxamidoadenosine

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LJ-529	2-Chloro-N^6-(3-iodobenzyl)-4′-thioadenosine-5′-methyluronamide
LJ-1251	(2*R*,3*R*,4*S*)-2-(2-Chloro-6-(3-iodobenzylamino)-9H-purin-9-yl) tetrahydrothiophene-3,4-diol
LJ-1416	(2*R*,3*R*,4*S*)-2-(2-Chloro-6-(3-chlorobenzylamino)-9H-purin-9-yl)tetrahydrothiophene-3,4-diol
LUF6000	*N*-(3,4-Dichloro-phenyl)-2-cyclohexyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine
Me	Methyl
MRS1292	(2*R*,3*R*,4*S*,5*S*)-2-[N^6-3-Iodobenzyl)adenos-9'-yl]-7-aza-1-oxa-6-oxospiro[4.4]-nonan-4,5-diol
MRS1760	(1'S,2'R,3'S,4'R,5'S)-4'-{2-chloro-6-[(3-iodophenylmethyl) amino]purin-9-yl}-1-(hydroxymethyl)bicyclo[3.1.0]hexane-2,3-diol
MRS1898	(1'S,2'R,3'S,4'R,5'S)-4'-{2-chloro-6[(3-iodophenylmethyl) amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0] hexane-2,3-diol
MRS3558	((1'R,2'R,3'S,4'R,5'S)-4-{2-Chloro-6-[(3-iodophenylmethyl) amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo-[3.1.0]-hexane-2,3-diol)
MRS5127	(1′R,2′R,3′S,4′R,5′S)-4′-[2-Chloro-6-(3-iodobenzylamino)-purine]-2′,3′-O-dihydroxybicyclo-[3.1.0]hexane
MRS5147	(1′R,2′R,3′S,4′R,5′S)-4′-[2-Chloro-6-(3-bromobenzylamino)-purine]-2′,3′-O-dihydroxybicyclo-[3.1.0]hexane
MRS5151	(1′S,2′R,3′S,4′S,5′S)-4′-[6-(3-Chlorobenzylamino)-2-(5-hydroxycarbonyl-1-pentynyl)-9-yl]-2′,3′-dihydroxybicyclo[3.1.0] hexane-1′-carboxylic acid *N*-methylamide
NECA	Adenosine 5′-*N*-ethyluronamide
PAMAM	polyamidoamine
QSAR	Quantitative structure–activity relationships
r	Rat
SARs	Structure–activity relationships
TNFα	tumor necrosis factor α
VUF5455	4-Methoxy-*N*-[7-methyl-3-(2-pyridinyl)-1-isoquinolinyl]benzamide

6.1 Introduction to A_3AR Agonists: Biological Effects and Therapeutic Prospects

Adenosine, a primordial signaling molecule present in almost every cell of the human body, mediates/regulates its physiological functions by interacting with four subtypes of G-protein-coupled receptors (GPCRs), termed A_1, A_{2A}, A_{2B} and A_3 (ARs) (Fredholm et al. 2001; Jacobson and Gao 2006). The A_3AR, although the least widely distributed in the body of the four AR subtypes, has distinct cytoprotective properties. Moreover, the A_3 subtype has been subject of intensive investigations as a potential therapeutic target and is perhaps the most enigmatic among the ARs since it displays a dual char-

acter in various circumstances under different patho-physiological conditions (Gessi et al. 2008). Although much remains to be learned about the function of the A_3AR, in particular about its duality, it is anticipated that A_3AR selective ligands might show considerable utility in the treatment of ischemic conditions (Strickler et al. 1996; Auchampach et al. 2003), glaucoma (Yang et al. 2005), asthma (Brown et al. 2008), cancer (Bar-Yehuda et al. 2008) and other disorders in which inflammation (Haskó et al. 2008) is a feature. In this context, development of novel strategies to alter and enhance the pharmacological profile of a given ligand would be of considerable utility. These characteristics have made agonist ligands of the A_3AR an attractive focus for medicinal chemical development. With the newly reported x-ray crystallographic structure of the A_{2A}AR (Jaakola et al. 2008), the prospect for achieving true structure-based ligand design for the A_{2A}AR and other ARs is evident.

The A_3AR is expressed in relatively high densities in lung, liver, neutrophils, macrophages, and glial cells. In the heart and brain, there are distinct effects on cell survival, but the A_3AR is expressed at a much lower level. Its presence on neurons in the central nervous system was controversial, but now well established (Lopes et al. 2003; Yaar et al. 2002).

Second messenger systems associated with the A_3AR have been studied. Activation of the A_3AR leads to inhibition of adenylate cyclase through coupling to G_i, and through the β,γ subunit it may also result in activation of phospholipase C (PLC), leading to a rise in intracellular calcium. Mitogen activated protein kinases (MAPKs) may also be activated by the A_3AR, resulting in effects on cell survival, proliferation, and differentiation (Schulte and Fredholm 2003). In an engineered CHO (Chinese hamster ovary) cell system, activation of human β-arrestin2 by the human (h) A_3AR was demonstrated (Gao and Jacobson 2008). The arrestin pathway could produce desensitization of a given GPCR, but is also known to have a signalling role. Therefore, multiple pathways of signal transduction are associated with the A_3AR, and assigning a specific pathway as the mechanism of a desirable effect of A_3AR agonists is not always straightforward. Evidence for biased agonism in nucleoside derivatives that favor one effector pathway over others, either in potency or in kinetic behavior, has been reported.

The A_3AR has a dual effect of inducing cytoprotection, particularly at low concentrations of agonist, and can also induce apoptosis and other cell death, particularly at high agonist concentrations. A_3AR agonists are currently in clinical trials, based on their anti-inflammatory and anticancer effects. Both of these applications depend on deregulation of the NFκB pathway (Bar-Yehuda et al. 2008). Activation of the A_3AR reduces the production of TNFα, which is well known as a damaging mediator in inflammatory conditions, such as rheumatoid arthritis. Other potential areas for application of A_3AR agonists are based on their cerebroprotective and cardioprotective effects. Among the first in vivo actions of the selective A_3AR agonist IB-MECA **7** (Fig. 6.1) were depression of locomotor activity and protection in a model of forebrain ischemia in gerbils. There was a difference between chronic and acute application of the A_3AR agonist in the cereboprotective model, leading to the proposal of an "effect reversal" phenomenon (Jacobson et al., 1996). Activation of cardiac A_3ARs preconditions cardiac myocytes against ischemic damage (Strickler et al. 1996; Tracey et al. 2003) and in some models induces protection during a period of prolonged ischemia. At least one A_3AR agonist, CP608039 **10** (Fig. 6.2), was under

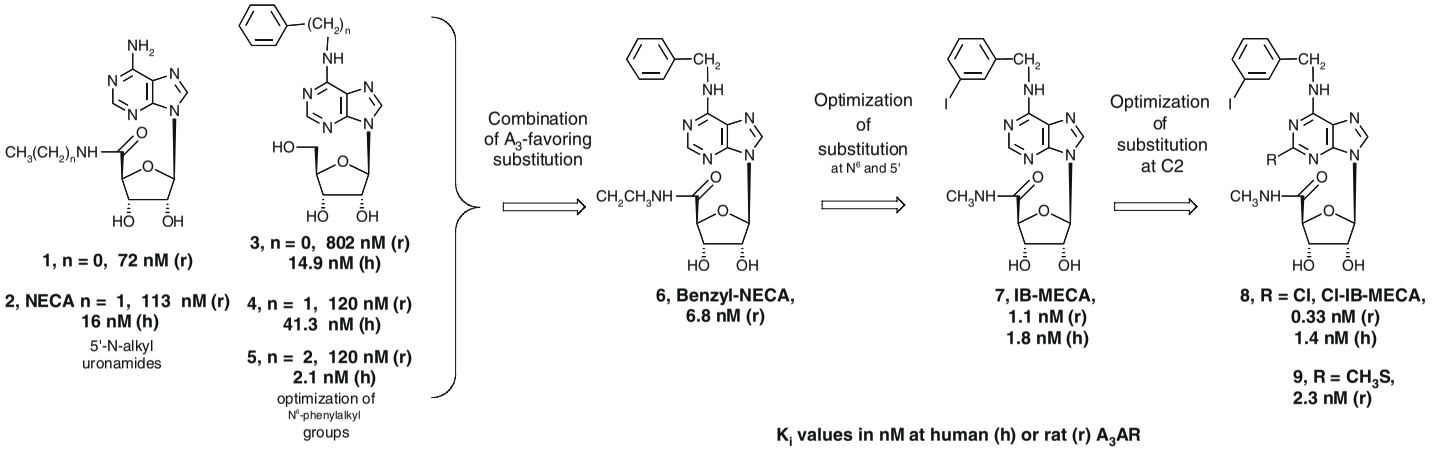


Fig. 6.1 Development of the SAR leading to the first selective agonist probes of the A_3AR

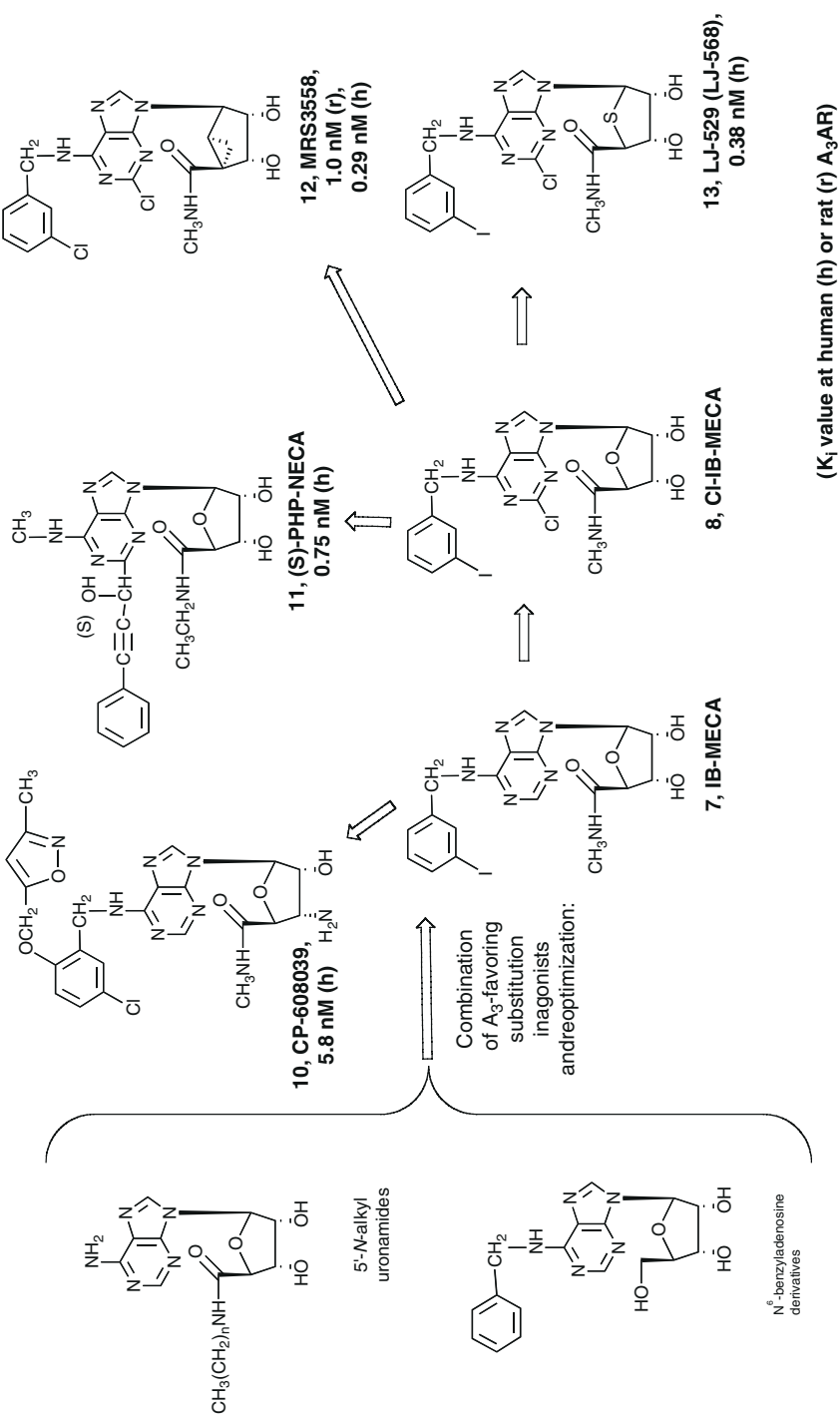


Fig. 6.2 Structural refinement of agonist probes of the A_3AR leading to enhanced selectivity

development as a preclinical candidate for this application. Especially intriguing is that A_3AR agonists maintain the function of mitochondria in cardiac muscle better than other AR agonists and protect against the cardiotoxic effects of the anticancer drug doxorubicin (Shneyvays et al. 2001).

In addition to the activation of the A_3AR by orthosteric agonists that are competitive with adenosine, allosteric enhancers of the action of adenosine and synthetic agonists at the A_3AR have been identified. The allosteric modulation of the A_3AR in vivo would depend on the release of endogenous adenosine in response to tissue or organ stress. Thus, it would amplify a natural protective response to a challenge.

The pharmaceutical development of A_3AR antagonists has also led to preclinical candidates for the treatment of glaucoma (Yang et al. 2005) and cancer (Gessi et al. 2008). It is striking that both A_3AR agonists and antagonists are being explored for cancer treatment. A_3AR antagonists will be covered in a separate chapter (Baraldi et al. this volume).

Nanotechnology has been applied to the study of ARs. We have recently demonstrated for the first time the feasibility to modulate the pharmacological profile of a ligand of a GPCR, specifically the A_{2A} and A_3ARs, based on conjugation to a polyamidoamine (PAMAM) dendrimeric nanocarrier (Kim et al. 2008). This finding raises the possibility of using dendrimer conjugates in tissue rescue from ischemia, and such macromolecular conjugates may help probe the existence of AR dimers.

6.2 A_3AR Agonists: First Leads and Essential Screening Tools

Agonist ligands for the A_3AR are almost exclusively nucleoside derivatives. The structure–activity relationship (SAR) of adenine nucleoside derivatives in A_3AR binding is now well-developed, with each position on the structure of adenosine suitable for derivatization having been extensively modified. Thus, nucleoside derivatives that are highly selective A_3AR agonists have been introduced. In the course of studying the SAR of adenosine derivatives at the A_3AR, it was discovered that variations in the relative efficacy are also commonplace (Gao et al. 2002b, 2003, 2004), in addition to variation in the binding affinity. This observation has led to the introduction of certain nucleoside derivatives as A_3AR antagonists.

6.2.1 Discovery of First A_3AR Agonists

The approach to initially identifying and structurally refining the first A_3AR agonists was to screen broadly libraries of nucleoside derivatives in binding assays at the newly-cloned A_3AR. The first report of an orphan receptor that resembled the known AR sequences was in fact the A_3AR of the rat (Meyerhof et al. 1991; Zhou et al. 1992). The rat (r) A_3AR and later the human (h) A_3AR were available for screening at NIH and elsewhere in the early 1990s, leading to the first A_3AR-selective agonists.

The species differences in affinity of these protypical A_3AR agonists were relatively modest, so the selectivity of most of the agonists identified using the rA_3AR later generalized to the hA_3AR. These libraries included both known AR ligands and those known to be weak or inactive at the other AR subtypes. Modifications of adenosine that were found to be conducive to activation of the A_3AR were then combined in the same molecule. The modifications of adenosine that were found to favor A_3AR affinity were at the N^6 position of adenine or at the 5′ position of the ribose group (Fig. 6.1), and these two sets of modifications could be combined to acheive an additive enhancement of affinity. For example, the agonist NECA **2** (adenosine 5′-*N*-ethyluronamide) was a highly potent A_3AR agonist, but not selective for this receptor subtype (Zhou et al. 1992). Modification of the N^6 position of adenine was studied systematically, and the favored group at this site for A_3AR selectivity was the N^6-benzyl group (Gallo-Rodriguez et al. 1994; van Galen et al. 1994). In comparison to the homologues in that series, that is, N^6-phenyl and N^6-phenylethyl, it was not an affinity enhancement that was key, but rather the fact that relative selectivity in comparison A_1 and A_{2A}ARs could be achieved. These structural features that promoted A_3AR potency were initially combined, resulting in N^6-benzyl-NECA **6**, which was 7-fold selective for the A_3AR.

Refinement of the substituents at the 5′ and N^6 positions led to the first selective A_3 agonist, IB-MECA **7** (N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine), introduced in 1993 (Jacobson et al. 1993). Thus, at the 5′ position, an *N*-methyluronamide favored the A_3AR better than an *N*-ethyluronamide, which was particularly potent at the A_{2A}AR. At the N^6 position, various substitutions of the aromatic ring of the N^6-benzyl group indicated that meta substitution was most desirable, and the halogens at this position were particularly favorable. This culminated in the introduction of the potent A_3AR agonist IB-MECA **7**.

A further refinement of IB-MECA was the introduction of substitution at the 2 position. Thus, the more selective 2-chloro analogue of IB-MECA, Cl-IB-MECA **8** (2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine), became a widely used pharmacological tool. This compound displayed a K_i of 0.33 nM at the rA_3AR, but a K_i of only 2,500 and 1,400 nM at rA_1 and A_{2A}ARs, respectively (Kim et al. 1994a). At the hARs, the binding affinities of Cl-IB-MECA were determined to be (nM): A_1AR 220, A_{2A}AR 5400, and A_3AR 1.4. Thus, Cl-IB-MECA is more selective for the rA_3AR than the hA_3AR (Melman et al. 2008a). IB-MECA **7** (CF101) and Cl-IB-MECA **8** (CF102) have entered Phase II clinical trials for the treatment of rheumatoid arthritis and cancer (Baharav et al. 2005; Ohana et al. 2001).

Among the four ARs, the species-dependence of ligand affinity is particularly pronounced for the A_3AR. Thus, medicinal chemical studies of the AR ligands should consider this important variable. Among agonists, this applied particularly for adenosine derivatives that contain small alkyl N^6 substituents and N^6-(2-phenylethyl) groups, which were shown to typically be more potent in binding at the hA_3AR than at the rA_3AR (Tchilibon et al. 2004). Nevertheless, many of other nucleoside analogues developed for this receptor, including various substituted N^6 – benzyl derivatives of adenosine tended to be selective for both the rA_3AR and the hA_3AR (Jacobson and Gao, 2006; Salvatore et al. 1993).

Early in the process of designing A_3AR agonists, the value of molecular modeling was recognized for structure-guided design (Kim et al. 2003). In the absence of an X-ray crystallographic structure of any of the ARs until recently, it was necessary to rely on homology modeling based on the structure of the light receptor, rhodopsin. The first molecular model of the A_3AR was reported in 2000, and included a docking hypothesis for adenosine agonists. Ligand docking models provided predictions concerning the environment of subregions within the putative binding site, which was used to interpret SAR and suggest modified ligands to be synthesized.

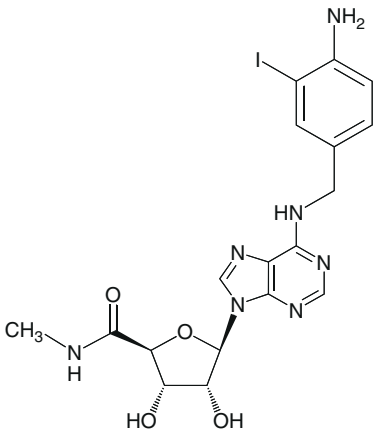
The putative binding site of the A_3ARs has been probed by site-directed mutagenesis to test the predicted modes of agonist docking made using molecular modeling (Kim et al. 2003; Gao et al. 2002c). A hydrophobic environment composed largely of TMs 5 and 6 and Phe168 of the second extracellular loop surrounds the purine ring and the large N^6 substituent of typical A_3AR agonists. The region surrounding the ribose moiety within the putative ligand binding pocket is lined with predominantly hydrophilic residues of TM3, such as Thr94, and TM7, such as Ser271 and His272. The exocyclic NH group is common to nearly all adenosine agonists acts as a putative H-bond donor to Asn250 in TM6. The availability of an agonist docking model has aided in the iterative process of ligand design and provided a source for new structural hypotheses to test using mutagenesis.

6.2.2 A_3AR Agonist Radioligands and Spectroscopic Probes

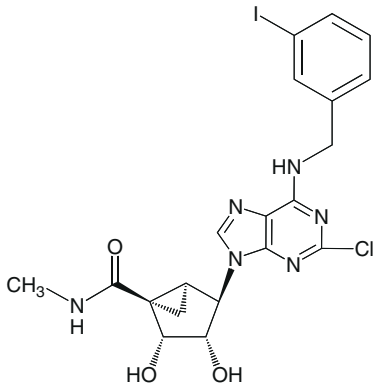
A derivative **14** related to IB-MECA is widely used as an iodinated radioligand for the A_3AR (Fig. 6.3). Thus, the ^{125}I form of I-AB-MECA (N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine, **14**) is commonly used as a high affinity radioligand for charcterization of binding to the A_3AR of various species. The selectivity of [^{125}I]I-AB-MECA for the A_3AR is low; it has also been used as a radioligand for the A_1AR. Therefore, it is most useful in transfected cell systems, in which the predominant AR present is the A_3AR.

A newer, more A_3AR-selective, agonist [^{125}I]MRS1898 **15** was recently introduced as a radioligand (Gao et al. 2009). This ligand was truly selective for the A_3AR vs. the A_1AR in human and rat, by 90- and 76-fold, respectively. Antagonist radioligands have also been reported and will be reviewed in another chapter (Baraldi et al. this volume).

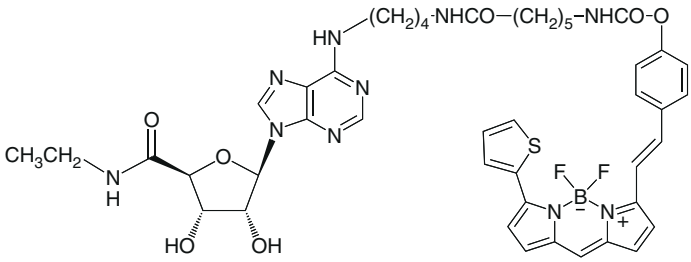
Agonist fluorescent probes that are A_3AR-selective have also been made by attaching 7-nitrobenzofurazan fluorophores to NECA derivatives using an alkyl spacer, for example, compound **16**. Also, a PAMAM dendrimer conjugate containing an N^6-derivatized adenosine derivative served as a fluorescent probe of the A_3AR. These compounds displayed high affinity and selectivity in binding to the A_3AR and therefore were useful in receptor detection and characterization in situ (Cordeaux et al. 2008).



14, I-AB-MECA



15, MRS1898



16, Bodipy conjugate

Fig. 6.3 Radioactive and fluorescent agonist probes of the A_3AR used in receptor detection and characterization and in compound screening

6.3 Detailed Structure Activity Relationship of Nucleosides as A_3AR Agonists

6.3.1 Modulation of Affinity

6.3.1.1 Modification of the Nucleobase

N^6-position of adenine. Substitution of the N^6 position of adenosine has been studied systematically (Fig. 6.4, **17-27**). It may induce selectivity for the A_1AR or A_3AR, or in some limited cases for the A_{2A}AR. N^6-Methyl **17** and N^6-methyloxy **18** substitution

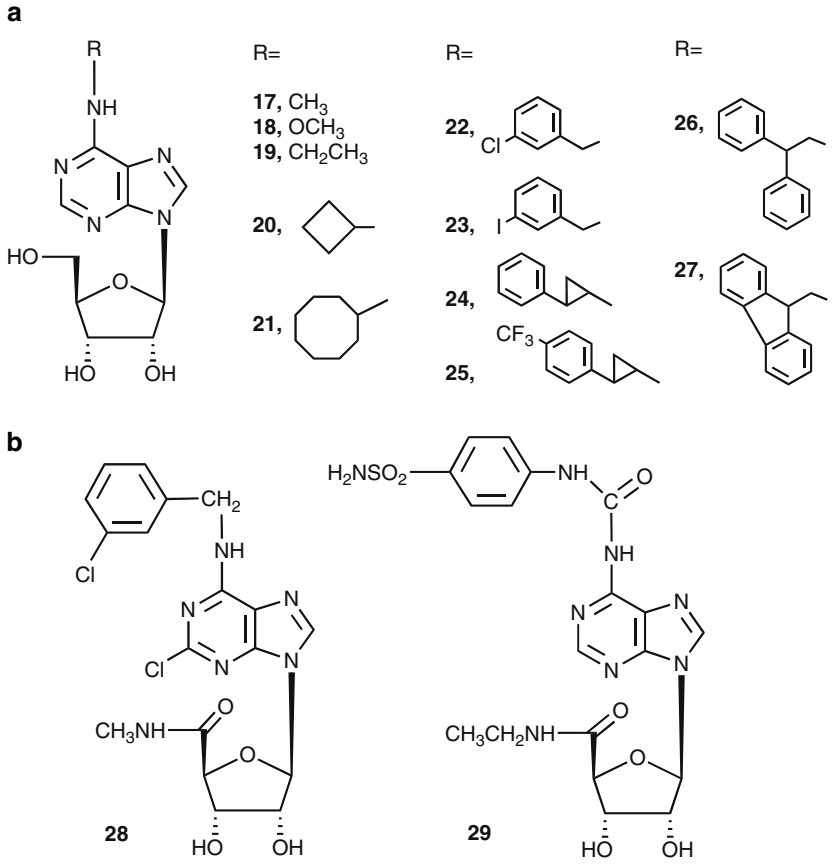


Fig. 6.4 N^6-substituted adenosine derivatives as agonists of the A_3AR

of adenosine tended to increase the potency and increased the selectivity of adenosine derivatives for the hA_3AR (Volpini et al. 2007), but this selectivity does not generalize to murine species. N^6-Ethyl substitution of adenosine in **19** increased the affinity at both A_3 and A_1ARs, thus, decreasing the selectivity (Gao et al. 2003). Larger alkyl chains at the N^6 position, both normal and branched, decreased A_3AR affinity and efficacy. There was a progression in the cases of N^6-cycloalkyl groups: from N^6-cyclobutyl **20** to N^6-cyclooctyl **21**, the relative efficacy as hA_3AR agonists decreased from 100% to 49% of a full agonist, with a decrease in A_3AR affinity.

Combination of aryl and alkyl groups on the N^6 substituent was also fruitful in the design of A_3AR agonists. Notably, N^6-benzyladenosine **4** (Fig. 6.1, K_i 41 nM) was three- to fourfold selective in binding to the hA_3AR in comparison to the A_1 and A_{2A}ARs, but it only displayed a 55% relative efficacy at the A_3AR. The lower homologue, N^6-phenyladenosine **3** (K_i 14.9 nM) was fully efficacious as an A_3AR agonist, but not as selective as N^6-benzyladenosine. The next higher homologue

N^6-(2-phenylethyl)adenosine **5** was more potent in binding to the hA_3AR (K_i 2.1 nM) than N^6-benzyladenosine but less selective due to its high affinity at the hA_1AR (K_i 12.9 nM).

Ring substitution of the N^6-benzyl moiety was also explored. Generally, halogen substitution at the 3 position resulted in increased A_3AR affinity and selectivity. N^6-(3-Chlorobenzyl)adenosine **22** (K_i 4.4 nM) and N^6-(3-iodobenzyl)adenosine **23** (MRS541, K_i 5.8 nM) (Fig. 6.4) were tenfold and 3.4-fold selective for the hA_3AR vs. the rA_1AR, respectively. Halogen substitution at other positions of the ring or substitution with nonhalogens at the 3 position typically decreased the A_3AR affinity in comparison to the above two compounds.

Variation of an N^6-(2-phenylethyl) substituent also provided A_3AR selective agonists. Rotational constraint in the form of a cyclopropyl group in N^6-(*trans*-2-phenylcyclopropropyl)adenosine **24** led to a full agonist with high potency and selectivity at the human, but not rA_3AR (Gao et al. 2003; Tchilibon et al. 2004). Addition of the p-CF_3 group to give N^6-(*trans*-2-(3-trifluoromethyl)phenyl)-1-cyclopropyl adenosine **25** resulted in a 100-fold selectivity at the A_3AR in comparison to the A_1AR. The addition of a second phenyl ring to the β-carbon of N^6-(2-phenylethyl)adenosine, that is, N^6-(2,2-diphenylethyl)adenosine **26** resulted in antagonism at the A_3AR, as determined in an assay of adenylate cyclase inhibition. Curiously, adding a bond between the phenyl groups to create N^6-(9-fluorenylmethyl) adenosine **27** provided full efficacy at the hA_3AR. This compound also displayed affinity of 0.91 nM at the hA_3AR, but was not very selective.

Baraldi et al. (1998) prepared a series of N^6-substituted-aminosulfonylphenyl derivatives of NECA, for example, compound **29** with a K_i value of 9.73 nM at the rA_3AR and selectivity of 47- and 120-fold vs. rA_1AR and $rA_{2A}AR$, respectively. Among these compounds, the most favorable substituents of the sulfonamido group for increasing affinity at the A_3AR were small alkyl groups, such as ethyl or allyl moieties, and disubstitution of the sulfonamido group. The A_3AR selectivity was increased by the addition of a saturated heterocyclic ring, such as piperidine or morpholine, to the sulfonamido moiety.

Thus, the combination of various sterically bulky N^6 groups with small *N*-alkyl-5′-uronamide moieties on the ribose was a fruitful approach to potent and selective A_3AR agonists. Another potent and selective agonist contains N^6-(3-chlorobenzyl) and 5′-methylcarboxamido groups **28** (Fig. 6.4).

C2-position of adenine. The 2 position of adenosine (Fig. 6.5, **30–36**) was earlier identified as a derivatization site for increasing $A_{2A}AR$ potency, but certain modifications have been found to produce or enhance A_3AR selectivity. 2-Chloroadenosine **30** displayed increased affinity in comparison to adenosine at both the the A_1 and A_3ARs (Gao et al. 2004). 2-Thioether modifications of adenosine that are not in combination with other modifications tend to reduce affinity at A_3AR. Note that in combination with other modifications, the 2-methythio group in **9** (Fig. 6.1) maintained high affinity and selectivity at the rA_3AR.

Adenosine analogues modified at the 2 position were studied systematically at the hA_3AR in binding and functional assays of adenosine 3′,5′-cyclic phosphate (cAMP) (Fig. 6.5). Certain 2-ether derivatives were particularly potent in binding

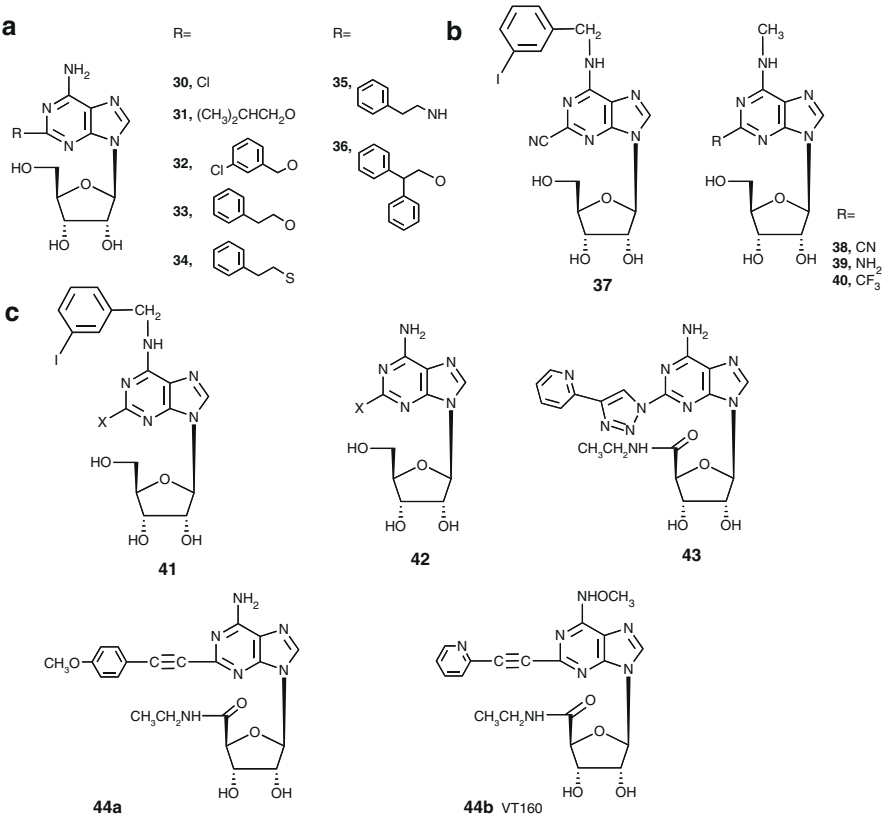


Fig. 6.5 C2-substituted adenosine derivatives as agonists of the A_3AR

to the A_3AR. Most modifications decreased A_3AR affinity, but some analogues had enhanced A_3AR affinity. For example, 2-*i*-butyloxy-adenosine **31** displayed a K_i value of 84 nM and was slightly (threefold) A_3AR selective. 2-(3-Chlorobenzyloxy)-adenosine **32** displayed a K_i value of 41 nM in binding to the A_3AR, but its relative efficacy was only 31% of the full agonist Cl-IB-MECA **8** (Fig. 6.1). A direct comparison was made between 2-ether, 2-thioether, or 2-amino substitutents (compounds **33**–**35**), and the oxygen analogue was clearly more potent. Thus, 2-phenylethyloxy-adenosine **33** and its various substituted analogues had increased affinity at both the A_3 and A_{2A}ARs, with high relative efficacy as A_3AR agonists. Conversely, some of the substituted phenylethyl derivatives, such as 2-(2,2-diphenylethyloxy)adenosine **36**, were A_3AR antagonists. Curiously, there was a parallel between effect of a given group (benzyl or phenethyl) when placed at the 2 position as an ether or at the N^6 position (Tchilibon et al. 2004). This suggests that there may be an overlap of these subregions in the A_3AR binding site.

The combined effects of 2-substitution and N^6 substitution of adenosine have been studied, and further illustrate the interrelatedness of these two positions (Fig. 6.5b). For example, Ohno et al. (2004) found different effects upon combining a 2-cyano group with either small or large N^6 substitution of adenosine. 2-Cyano-N^6-(3-iodobenzyl) adenosine **37** was an A_3AR antagonist, and 2-cyano-N^6-methyladenosine **38** was a full agonist that was 30-fold selective for the hA_3AR in comparison to the A_1AR. However, the 2-amino **39** and 2-trifluoromethyl **40** derivatives of N^6-methyladenosine were considerably weaker in A_3AR binding.

Heterocyclic aryl groups have been placed at the 2 position of adenosine and its derivatives. For example, Elzein et al. (2004) synthesized a series of 2-pyrazolyl-N^6-substituted adenosine derivatives, such as **41**, that were very potent and selective for the A_3AR. Cosyn et al. (2006b) found that several 2-triazol-1-yl substitutions of N^6-methyladenosine increased affinity at the A_3AR. The 5′-CH_2OH (ribose-like) derivatives, such as **42**, tended to display less than full efficacy at the A_3AR, but a 5′-ethyluronamide group in the 2-(4-pyridin-2-yl-1,2,3-triazol-1-yl)adenine derivative **43** restored full agonism in analogous compounds. Thus, compound **43** was a full agonist with a K_i of 1.8 nM at the A_3AR and a minimum of 900-fold selectivity over other ARs.

The combined effects of 2-substitution and 5′ substitution of adenosine have been studied. Additions at the 2 position of NECA often increased potency and/or selectivity. For example, 2-(3-hydroxy-3-phenyl)propyn-1-yl-NECA **11** (PHPNECA) (Fig. 6.2) was particularly potent at the A_3AR but nonselective (Volpini et al. 2002). Another analogue 2-(2-phenyl)ethynyl-NECA (PENECA) displayed a higher selectivity for the A_3AR (100-fold, K_i = 6 nM). Methylation at the N^6 position increased selectivity in the 5′-CH_2OH series, as in 2-phenylethynyl-N^6-methyladenosine, which showed an A_3AR affinity in the low nanomolar range (K_i = 3.4 nM) and high subtype selectivity. The most potent compound in that series **44a** (Fig. 6.5) had a (*p*-(methoxy)phenyl)alkynyl substituent at the 2 position.

Recently, new potent and A_3 selective N^6,2-disubstituted adenosine derivatives have been reported. Volpini et al. (2007) made a series of N^6-methoxy-2-alkyladenosine derivatives, of which **44b** was the most potent and selective. This compound had a K_i of 2.5 nM at the hA_3AR and selectivity of 21,000 and 4,200 against A_1 and A_{2A}ARs respectively.

Nonadenine Nucleobases. A few non-adenine nucleotides, notably xanthines-7-ribosides, have been found to activate the A_3AR. Although the parent 1,3-dialkylxanthine acts as an AR antagonist, addition of a sugar moiety to the 7 position (rather than 9 position of the purine moiety in the standard nucleosides) results in A_3AR agonism. For example, *N*-methyl-1,3-dibutylxanthine 7-β-D-ribofuronamide **45** (DBXRM, Fig. 6.6) acted as a moderately selective A_3AR agonist (Kim et al. 1994b). Curiously, the degree of relative efficacy depended on the point in the signalling cascade that was measured. At the rA_3AR, by the criterion of adenylate cyclase inhibition DBXRM was a full agonist, while using the functional assay of [^{35}S]GTPγS binding DBXRM was a partial agonist. Thio modification in the xanthine-7-riboside series, as in **46**, was well tolerated at the rA_3AR.

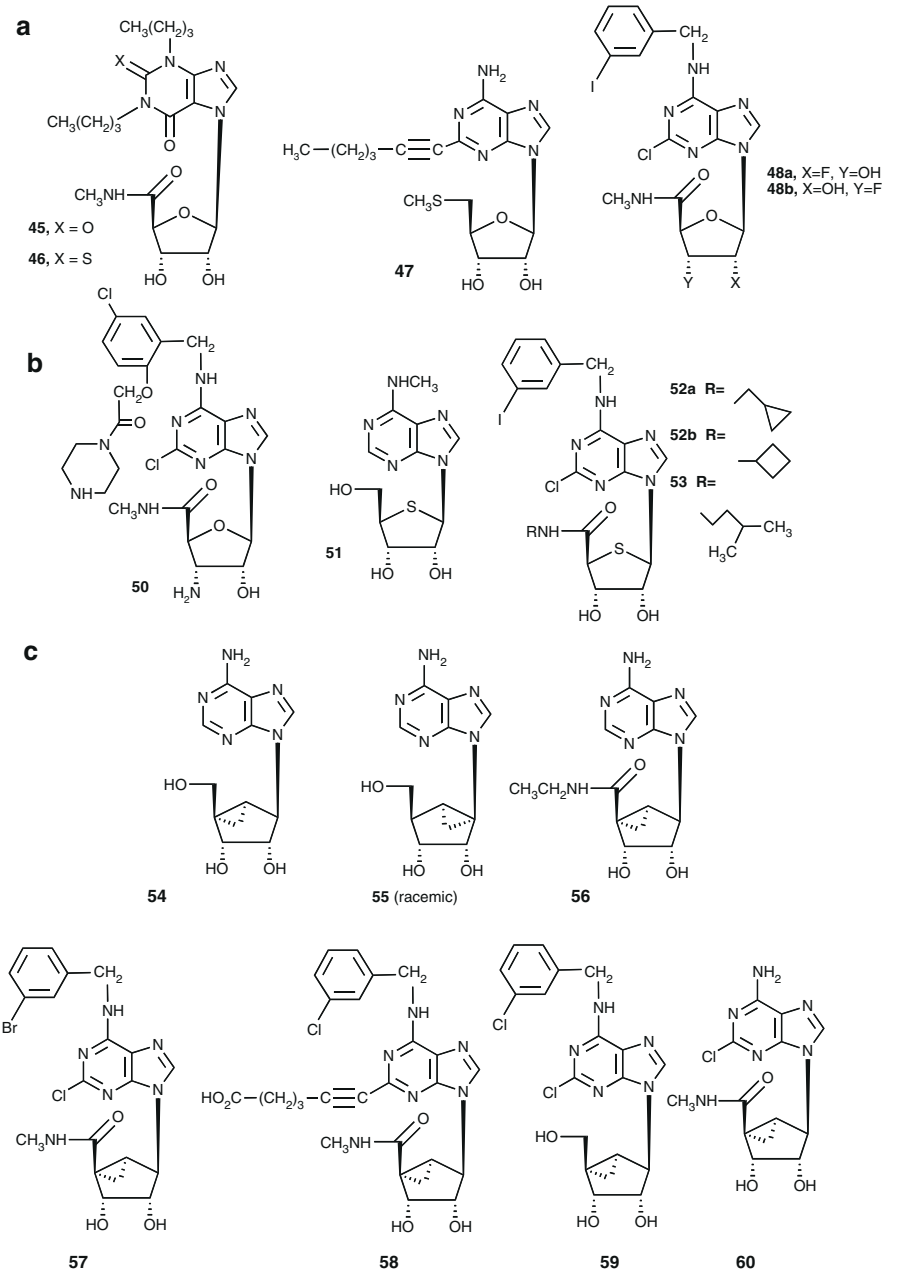


Fig. 6.6 Ribose-modified adenosine derivatives as agonists of the A_3AR

6.3.1.2 Modification of the Ribose Moiety

Modifications of the ribose moiety (Fig. 6.6, **47–60**) have also been explored for their effect on both A_3AR binding affinity and efficacy (Gao et al. 2004; van Tilburg et al. 2002). The 5′-*N*-alkyluronamide modification with small alkyl groups, such as **1** and **2** (Fig. 6.1), was conducive to enhanced affinity at both the A_{2A}AR and A_3AR. With proper combination with other modifications, selectivity for the A_3AR could be achieved. Other modifications at the ribose 5′ position, such as alkylthioethers (van Tilburg et al. 2002) were found to modulate both affinity and efficacy at the the A_3AR. Thus, the 2-hexynyl-5′-thioether derivative **47** has a K_i value of 14.5 nM at the hA_3AR and only 72% relative efficacy in inhibition of cAMP production.

Both the 2′- and 3′- hydroxyl groups are critical for both binding and activation functions at the A_3AR, and these effects appear to be related to H-bond donation to the receptor protein. Replacement of either of these groups in Cl-IB-MECA with a fluoro group (in **48** and **49**), which can only accept but not donate a H-bond, caused a greatly reduced both affinity and efficacy (Gao et al. 2004). The 2′-hydroxyl group appeared to be more important than the 3′-hydroxyl group, because the 2′-fluoro analogue **48** displayed no binding or activation of the A_3AR. However, in N^6-substituted adenosine analogues, replacement of the 3′-hydroxyl group with an amino group was tolerated (DeNinno et al. 2003, 2006), leading to A_3AR-selective agonists such as CP-608039 **10** (Fig. 6.2). DeNinno et al. recently reported the water-soluble 3′-amino-3′-deoxy nucleoside derivative **50** (50 – 100 mg/mL at pH 4) as a potent/selective A_3AR agonist with a K_i of 10 nM. Thus, when the 5′ and N^6 positions of adenosine were also appropriately modified, 3′-amino substitution gave high selectivity for the A_3AR (Fig. 6.3). Van Rompaey et al. (2005) found that various analogues of 3-amino-3-deoxyadenosine displayed moderate affinity at the A_3AR, but these compounds were only partial agonists. For example, the 3′-amino-3′-deoxy analogue of N^6-(5-chloro-2-methoxybenzyl)adenosine 5′-*N*-methyluronamide was a selective A_3AR partial agonist (K_i 27 nM, 51% relative efficacy). Cosyn et al. (2006a) also made a series of 3′-amino-3′-deoxy derivatives of adenosine, which were highly selective for the A_3AR. When a methylene spacer was added between the 3′-amino and ribose groups, there was a total loss of affinity at the native A_3AR (Van Rompaey et al. 2005), suggesting that there are restrictive geometric factors in this region of the binding site. 3′-Deoxy-3′-acetylamino analogues tend to bind only weakly at the A_3AR leading to partial activation, and replacement of the 3′-hydroxyl with an azido group generally abolished A_3AR activation.

4′-Thio derivatives (i.e., the ring oxygen is replaced with sulfur) were usually equipotent or slightly more potent at ARs than their oxygen equivalents (Jeong et al. 2003, 2006b). Many 4′-thio derivatives of adenosine such as **51** have been found to be full A_3AR agonists. A series of potent A_3AR agonists, partial agonists, and antagonists were made by varying the N^6 and 5′ groups. Adding an N^6 methyl group and 2-chloro group to 4′-thioadenosine-5′-methyluronamide created a compound with a K_i of 0.28 nM and nearly a 5,000-fold selectivity for the hA_3AR

(Jeong et al. 2003). LJ-529 **13** (Fig. 6.2) is a highly potent and selective agonist of both human (K_i = 0.38 nM in binding) and rA_3ARs. However, when the thio modification was combined with shifting the adenine moiety of Cl-IB-MECA from the 1′ to the 4′ position of the ribose ring, the compound was curiously transformed into a potent antagonist (Gao et al. 2004).

In the same 4′-thio modified series, a wide variety of ribose 5′-alkyluronamides have shown that there is tolerance for groups larger than *N*-ethyl (Jeong et al. 2006a). 4′-Thio adenosine analogues modified as 5′-amides were also studied systematically in functional cAMP assays at the hA_3AR. Various 5′-amide modifications decreased A_3AR efficacy, while retaining A_3AR affinity. For example, compounds **52a** and **52b** were full agonists at the A_3AR with K_i values of 3.6 and 18 nM, respectively, while **53** was a partial agonist (12% efficacy, K_i 41.9 nM). Thus, the nature of the *N*-alkyl or *N*-arylalkyl group can modulate both affinity and efficacy at the the A_3AR.

SAR studies also indicate that flexibility in the ribose 5′-region is a prerequisite for A_3AR activation, in concert with a proposed required rotation of TM6 of the A_3AR (Kim et al. 2006). This insight has allowed the conversion of nucleoside analogues that were at the boundary of agonist/antagonist into full antagonist at the A_3AR by structural modification. The modifications typically are made on the N^6 and/or ribose moieties, but modifications at the 2 position are also known to reduce the relative efficacy to the point of reduced residual ability to activate the A_3AR, thus providing an antagonist. A freely rotating 5′-uronamide that is able to make and break multiple H-bonds provides a necessary degree of flexibility of the ligand during the receptor activation step. There seems to be an order of dominance of modification at various positions with respect to their effect on relative efficacy at the A_3AR. For example, a flexible 5′-uronamide moiety, which occur in NECA and IB-MECA, generally overcomes the reduction of efficacy induced by other modifications at the N^6 and 2 positions (Gao et al. 2002b). However, a 5′-uronamide moiety was unable to overcome the reduction of efficacy resulting from substitution of the 3′-OH group (Gao et al. 2004).

Ring-constrained nucleosides have been used to define conformational preferences at the A_3AR. By conformationally constraining the otherwise freely-rotating ribose moiety, it is possble to gain an entropic contribution to the binding energy, thus raising the afffinity. Moreover, inactive conformations can be excluded due to inactivity at a given receptor. Nucleoside analogues containing novel rigid carbocyclic ring systems in place of the ribose ring have been explored as ligands for the A_3AR. The methanocarba (bicyclo[3.1.0]hexane) ring system has been incorporated into nucleoside analogues in either of two isomeric forms that adopt either a North (N) or South (S) envelope conformation (Jacobson et al. 2000; Marquez et al. 1996). (N)-Methanocarba-adenosine **54** was found to favor binding at the A_3AR, and this modification also demonstrated that the ring oxygen is not required for binding or activation of the receptor (Lee et al. 2001). In comparision to adenosine itself, the degree of affinity enhancement was 2.5-fold, and there was 150-fold enhancement in comparison to its isomeric derivative **55** locked in the (S) conformation. The (N)-methanocarba modification was subsequently combined with known enhancing modifications at other positions on the molecule to explore the resulting

SAR. Introduction of a (N)-methanocarba modification to NECA in **56** increased the hA_3AR binding affinity by sixfold. Thus, the (N) conformation is confirmed to be highly preferred over the (S) conformation for binding at the A_3AR, for a range of structures of multiply modified nucleoside derivatives.

Even more highly selective ring-constrained A_3AR agonists have been designed and synthesized based on the (N)-methanocarba ring system (Fig. 6.2). A series of (N)-methanocarba-2,*N*^{6}-disubstituted adenine nucleosides was made by Tchilibon et al. (2004), who found that combining the (N)-methanocarba modification with 2-chloro and 5′-methyluronamido groups significantly improved the selectivity and efficacy over the riboside derivatives. Among these agonists is MRS3558 **12**, which is a full agonist with subnanomolar potency at the A_3AR. Other 3-halo derivatives in the same series, for example, the 3-iodo derivative MRS1898 **15** and the 3-bromo derivative MRS3581 **57**, are similarly full agonists with nanomolar potency at the A_3AR (Tchilibon et al. 2005; Melman et al. 2008a). Compound **57** was recently labeled with ^{76}Br for use as a radiotracer in positron emission tomography. The utility of MRS3558 **12** (Fig. 6.2) in treating lung injury was shown in a model of ischemia reperfusion lung injury (Matot et al. 2006).

The species-dependence of (N)-methanocarba nucleosides at the A_3AR was explored. MRS3558, although highly selective at the hARs and moderately selective at the rARs, was found to be only tenfold selective for the mouse A_3AR. In general, the selectivity at the mouse A_3AR of analogues containing the (N)-methanocarba ring system was reduced due to an increased tolerance of this ring system at the mouse A_1AR (Melman et al. 2008a). However, substitution of the 2-chloro atom with iodo or hydrophobic alkynyl groups tended to increase the A_3AR selectivity (up to 430-fold) in mouse and preserve it in human. Elongation of an alkynyl chain at the 2 position remediated this reduced selectivity in the mouse and preserved A_3AR selectivity more effectively than similar chains attached at the 3 position of the *N*^{6}-benzyl group. For example, the 2-alkynyl carboxylic acid derivative MRS5151 **58** is a highly potent agonist (K_i = 2.38 nM at hA_3AR) and selective in binding at human (6,260-fold) and mouse (431-fold) A_3ARs in comparison to A_1ARs in the same species.

The effects of this ring constraint on efficacy at the A_3AR have also been described. Addition of a 5′-uronamide moiety generally assures that an appropriately derivatized (N)-methanocarba nucleosides will be a full agonist at the A_3AR. Some of the 5′-CH_2OH derivatives in this series, such as MRS1760 **59**, are full antagonists of the A_3AR.

Differential effects of A_3AR agonists in the inhibition of adenylate cyclase, stimulation of [^{35}S]GTPγS binding, and translocation of arrestin, and other effector systems may be compared (Gao and Jacobson 2008). There was evidence for biased A_3AR agonism in a comparison of potencies of various nucleoside derivatives in an arrestin-translocation response and their ability to inhibit adenylate cyclase. Also, in kinetic experiments, differences were seen. NECA and MRS3558 **12**, for example, displayed a rapid arrestin response, while IB-MECA **7** and Cl-IB-MECA **8** displayed a slower response. Differences in the functional potencies of the same analogue were also seen, depending on whether the response was G protein-dependent or independent. This is also suggestive of biased agonism.

6.3.2 Modulation of Efficacy by Nucleoside Modification

Interestingly, certain modifications, such as a 5′-alkylamide or an N^6-methyl group can restore efficacy to previously modified compounds. For instance, adding a 2-chloro group to N^6-cyclopentyladenosine creates an A_3AR antagonist (Gao et al. 2002b), but activation is restored by the 5′-methylcarboxamide and 4-thio substitutions. This is particularly interesting since 4′-thioadenosine is also an A_3AR antagonist, and 2-chloro-4-thioadenosine is only a partial agonist (Jeong et al. 2006b).

The effects on efficay of modifications at different positions of adenosine are interdependent. For example, the 2-cyano derivative of N^6-(2-phenylcyclopropyl) adenosine **24** was a hA_3AR antagonist, but its substitution with a N^6-methyl group restored full agonism (Ohno et al. 2004). N^6-*trans*-(2-Phenylcyclopropyl) adenosine **24** (Fig. 6.4) itself was a full A_3AR agonist. N^6-(2,2-Diphenylethyl)adenosine **26** was an A_3AR antagonist with 12-fold and 130-fold selectivity over A_1 and A_{2A}ARs, respectively. However, the combination with (N)-methanocarba, 2-chloro, and 5′-methyluronamido substitutions in **60** provided a full agonist with a K_i of 0.69 nM and a selectivity of close to 2,000-fold over A_1 and A_{2A}ARs (Tchilibon et al. 2004).

The sytematic conversion of nucleoside agonists of the A_3AR into selective antagonists is outlined in Fig. 6.7. Truncation of the 5′-alkylamide or its conformational constraint in the form of a siprolactam in MRS1292 **61** has been shown to accomplish this effect. The truncation of 4′-thio nucleosides (to give **63** and **65**) and the natural 4′-oxo nucleosides (to give **62** and **64**) was effective in providing selective A_3AR antagonists, as judged in cAMP assays (Jeong et al. 2008; Pal et al. 2009). High affinity and selectivity of binding to the A_3AR in both rat and human is preserved in these truncated nucleosides. In the (N)-methanocarba series, truncation of the 5′-methylamide of the known A_3AR-selective agonists provides compounds, such as **66** and **67**, that appear to be antagonists in functional assays of [^{35}S] GTPγS binding (Melman et al. 2008b), but display considerable efficacy when examined in assays of A_3AR-mediated modulation of adenylate cyclase.

6.4 Non-nucleoside (e.g., Pyridine) A_3AR Agonists

Although most AR agonists have been from the modification of the endogenous agonist, adenosine, a series of non-adenosine agonists has recently been synthesized and demonstrated to be potent agonists at ARs. A series of pyridine-3,5-dicarbonitriles derivatives has been shown to be agonists for the A_1AR (Chang et al. 2005). Some derivatives, such as BAY-60-6583, selective for the A_{2B}AR have also been synthesized (Eckle et al. 2007). A number of these derivatives were also found to be potent agonists of the A_3AR (Beukers et al. 2004). Although nonselective with respect to other ARs, compound **68** (Fig. 6.8) has a K_i value of 24 nM at the hA_3AR and is a partial agonist in the GTPγS binding assay (Gao et al. 2008).

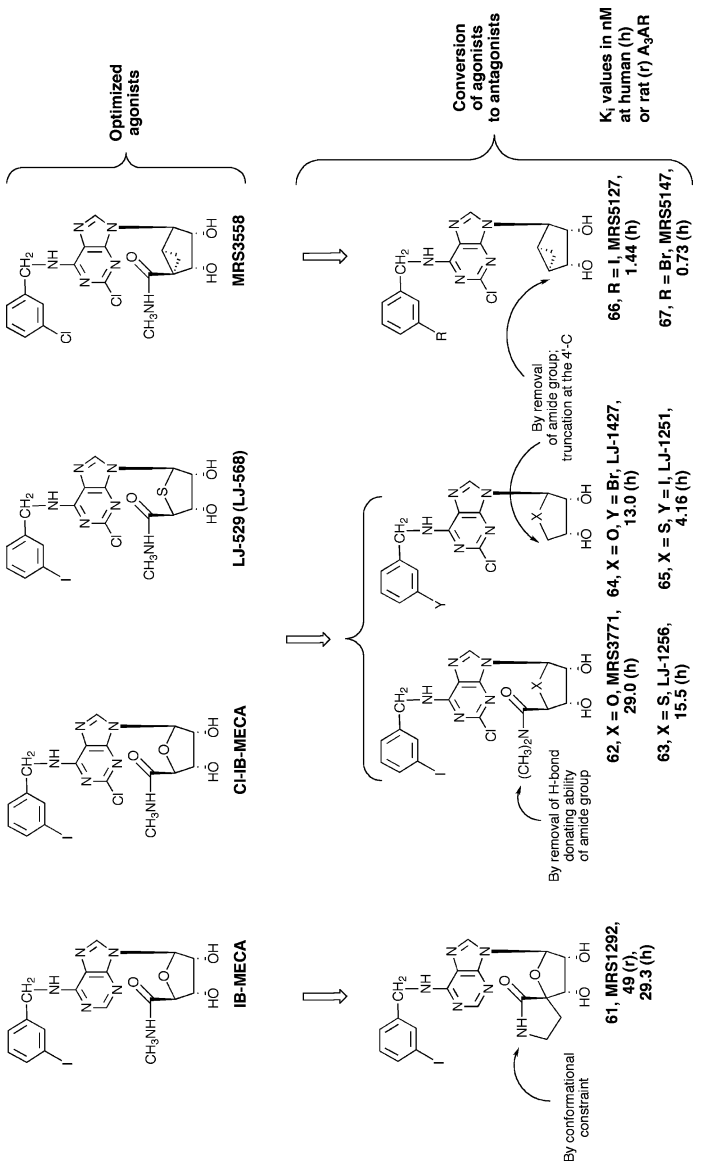
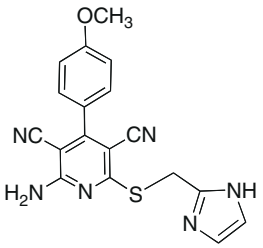


Fig. 6.7 Conversion of adenosine derivatives into selective antagonists of the A_3AR

Fig. 6.8 A pyridine derivative as an atypical (nonselective) agonist of the A_3AR



68, pyridine agonist (BAY-60-6583)

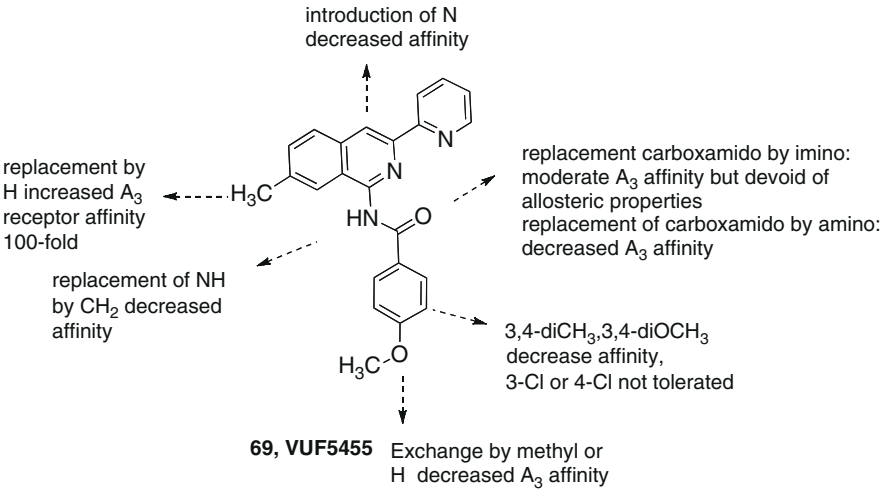


Fig. 6.9 Summary of known SAR of derivatives of VUF5455 as selective allosteric enhancers of the A_3AR

6.5 Allosteric Modulation of A_3AR Agonist Affinity and Efficacy

Like other GPCRs, the A_3AR possesses allosteric binding sites that are spatially and often functionally distinct from the orthosteric sites. Allosteric agonists, antagonists, and inverse agonists for a given GPCR will bind to the allosteric site and induce a similar effect as their orthosteric relatives (Christopoulos 2002; Gao et al. 2005). Allosteric modulators of a GPCR are those ligands that bind to an allosteric site to stabilize a receptor conformation and shift the equilibrium to increase or decrease the affinity and/or efficacy of an orthosteric agonist at the receptor. In theory, they do not activate the receptor on their own, although allosteric agonists are also known. The modulator increases (positive allosteric modulators, PAMs) or decreases (negative allosteric modulators, NAMs) signaling via the receptor only in the presence of an orthosteric agonist.

At the hA_3AR, a 3-(2-pyridinyl) isoquinoline **69** (VUF5455, Fig. 6.9) (Gao et al. 2001) and a 1*H* imidazo[4,5-*c*]quinolin-4-amine **70** (DU124183, Fig. 6.10)

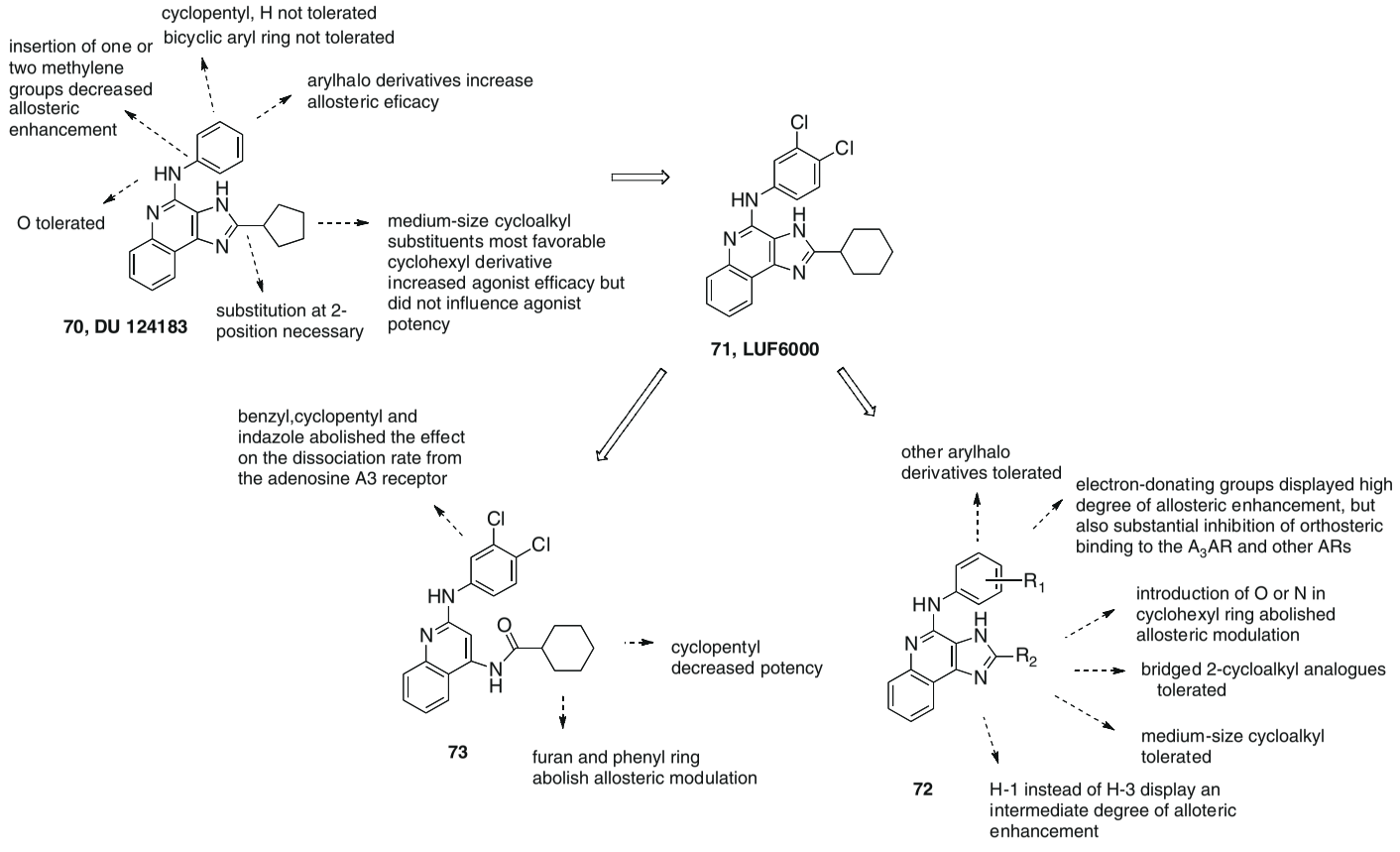


Fig. 6.10 Summary of known SAR of derivatives of DU124183 and LUF6000 as selective allosteric enhancers of the A_3AR

(Gao et al. 2002) were shown to be allosteric enhancers. VUF5455 and its congeners (Fig. 6.9), which were originally synthesized as A_3AR antagonists by IJzerman and colleagues, were reported by Gao et al. (2001) as the first selective allosteric enhancers of this receptor. The effects of these derivatives were examined on the dissociation rate of a high affinity A_3AR agonist radioligand, [^{125}I]I-AB-MECA (the degrees of dissociation at 60 min with agonist alone or in the presence of 10 μM modulator were compared). When the modulator slowed the dissociation rate of the radioligand, it indicated an allosteric enhancement of the agonist effect, assuming that association rate was not similarly affected.

The SAR of the 3-(2-pyridinyl) isoquinoline series show that the replacement of the 7-methyl group by H increased the A_3AR affinity by approximately 100-fold (K_i = 17.3 nM) without an effect on the allosteric activity. Introduction of a *N* at the 4 position decreased the affinity by ~sixfold in the parallel series. In the chain at the 9 position, replacement of the carboxamido group by an imine resulted in moderate A_3AR affinity in orthosteric binding (K_i values 300–700 nM), but this analogue lacked allosteric properties. The replacement of the NH by a methylene group in the chain decreased the affinity (K_i = 660 nM). A chloro substituent at the 3′ or 4′ position reduced the A_3AR affinity (K_i values of 200 and 770 nM, respectively). Exchanging the 4′-methoxy group by a methyl or H lowered the A_3 receptor affinity (K_i values of 96 and 204 nM, respectively) without affecting the allosteric activity. The introduction of a second methoxy group at the meta position in this ring decreased the A_3AR affinity (K_i = 310 nM).

A second series of allosteric modulators for the A_3AR that included DU124183 **70** (Fig. 6.10), synthesized originally as A_1AR antagonists by van Galen et al. (1991), were identified in the course of screening as allosteric modulators for A_3 receptors (Gao et al. 2002a). Similar to the 3-(2-pyridinyl)isoquinoline derivatives, several of these compounds selectively decreased the dissociation rate of the agonist [^{125}I]I-AB-MECA from the hA_3AR. The first leads from these analogues were the 2-cyclopentyl-1H-imidazo[4,5-*c*]quinoline derivatives, of which the 4-phenylamino analog DU124183 **70** had the most favorable degree of allosteric modulation (174% of control in the [^{125}I]I-AB-MECA dissociation) versus receptor antagonism. The SAR of this series (Göblyös et al. 2006) (Fig. 6.10) showed that insertion of one or two methylene groups at the 4 position decreased allosteric enhancement (145–154% of control in the [^{125}I]I-AB-MECA dissociation). Chloroaryl groups at the 4 position decreased the competition for orthosteric binding but retained the allosteric activity. Substitution at the 2 position is necessary for the activity (91% of control in the [^{125}I]I-AB-MECA dissociation). At this position, medium-size cycloalkyl substituents (cyclopentyl and cyclohexyl) were the most favorable for enhancement. Analogues bearing smaller or larger rings or an acyclic alkyl group were considerably less enhancing, and similarly an aromatic five-membered ring was not conducive to enhancement. As a result of this study, LUF6000 **71** (173% of control in the [^{125}I]I-AB-MECA dissociation) was identified as a novel allosteric enhancer of the A_3AR, because it had improved allosteric over orthosteric properties.

Recently, we published an extension of this study by modifying the substitutions around the 4-arylamino and 2-cycloalkyl moieties of the imidazoquinoline scaffold, as shown in structure **72** (Kim et al. 2009). At the 4 position, other arylhalo derivatives were tolerated (171–209% of control in the [^{35}S]GTPγS binding versus 208% for LUF6000). Introduction of electron-donating groups produced a high degree of allosteric enhancement, but also a substantial inhibition of orthosteric binding at the A_3AR and other ARs. The replacement of the phenylamino group by 3-pyridinylamine resulted in a low degree of allosteric enhancement.

Highly variable biological activities were observed for the 2 position derivatives. Replacement of the distal methylene group of the 2-cyclohexyl ring with an ether oxygen in the tetrahydropyranyl derivative abolished allosteric modulation of the A_3AR (114 % of control in the [^{35}S]GTPγS binding), but retained a similar degree of orthosteric inhibition. Introduction of a nitrogen atom in (or as a substitute of) the six-membered ring reduced the A_3AR allosteric enhancement of this series (96–111% of control in the [^{35}S]GTPγS binding). Inclusion of a methylene bridge across the 2-cyclohexyl ring resulted in considerable allosteric potentiation (156–210% of control in the [^{35}S]GTPγS binding). Thus, as extension of previous findings, the allosteric and orthosteric inhibitory effects at the A_3AR in this series of imidazoquinolines were structurally separable.

Heitman et al. (2009) recently reported a novel chemical template (**73**) based on LUF 6000. The imidazoquinoline heterocyclic ring system of LUF6000 was opened to give 2,4-disubstituted quinolines (Fig. 6.10). As in the previous series, a cyclopentyl, benzyl or indazole substituent abolished the effect on the rate of dissociation from the A_3AR (102–114% of control in the [^{125}I]I-AB-MECA dissociation). Again, the most potent allosteric enhancer was obtained with a 3,4-dichlorophenyl group at the 4 position (249 % of control in the [^{125}I]I-AB-MECA dissociation). At the 2 position, introduction of a cyclobutyl or cyclopentyl (119% and 145% of control in the [^{125}I] I-AB-MECA dissociation, respectively) instead of cyclohexyl resulted in compounds with lower modulating potency. Introduction of a furyl or phenyl ring at the 2 position abolished the allosteric enhancement (96% and 112% of control in the [^{125}I]I-AB-MECA dissociation respectively). An aromatic instead of an aliphatic substituent at this position was not tolerated.

Notably, the most potent enhancers of these series (Fig. 6.10) showed the highest structural similarity to LUF6000. Moreover, this compound showed a decreased orthosteric effect on the A_1 and A_3ARs when compared to LUF6000 **71**.

6.6 Conclusions

The dual personalities of the A_3AR often come into direct conflict, for example, in ischemia, inflammation and cancer, rendering this receptor as a single entity behaving in two different ways. The biology of the A_3AR can now be explored effectively with the availability of numerous selective agonists, with varying degrees of efficacy,

and enhancers. Structure-based ligand design based on crystallographic data is likely to be used in future medicinal chemical studies of the A_3AR. Biased agonists that favor one effector pathway over others will be developed for use in future studies of disease states.

IB-MECA, the first selective A_3AR agonist, was reported in 1993, and since then numerous adenosine derivatives have been modified to optimize their interaction with the A_3AR. IB-MECA (CF101) and its 2-chloro analogue, Cl-IB-MECA (CF102) are in Phase II clinical trials for the treatment of autoimmune inflammatory diseases and cancer, respectively. Additional structural modifications made at the N^6 and 2 positions and in the ribose moiety of adenosine derivatives have led to even more potent and selective A_3AR agonists. A newer generation of A_3AR agonists incorporate the (N)-methanocarba ring system in place of the ribose moiety, leading to increased affinity and subtype selectivity. The affinity of certain nucleoside derivatives at the A_3AR has been found to be species-dependent. Thus, the 2-alkynyl (N)-methanocarba derivative MRS5151 **58** (Fig. 6.6) was highly selective for both the mouse and the hA_3AR.

Differential effects of A_3AR agonists in the inhibition of adenylate cyclase, stimulation of [^{35}S]GTPγS binding, and translocation of arrestin, and other effector systems may be compared. Combinations of efficacy-lowering substitutions can convert agonists into partial agonists and even A_3AR antagonists. The role of each effector pathway in disease states will have to be established. Biased agonists can be designed to target specific diseases.

Positive allosteric modulators, such as imidazoquinoline derivatives, can enhance the relative efficacy of partial agonists of the A_3AR. For example, the efficacy of Cl-IB-MECA **8** (Fig. 6.1) in stimulating guanine nucleotide binding may be increased by the imidazoquinoline LUF6000 **71** (Fig. 6.10) and its analogues. The SAR of such allosteric modulators has been explored in detail. The allosteric modulation of the A_3AR has a particular appeal for therapeutics due to the site-specific and event-specific nature of this pharmacological intervention.

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Chapter 7
A_3 Adenosine Receptor Antagonists: History and Future Perspectives

Pier Giovanni Baraldi, Romeo Romagnoli, Giulia Saponaro, Stefania Baraldi, Mojgan Aghazadeh Tabrizi, and Delia Preti

7.1 Introduction

Adenosine exerts a number of physiological functions through activation of four cell membrane G-protein-coupled receptors classified as A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al. 2001).

A_3 AR subtype has been subject of intensive investigations as potential therapeutic target due to its contribution to important pathophysiological processes such as inflammation, neurodegeneration, ischaemia, asthma, glaucoma and cancer (Jacobson 1998; Gessi et al. 2004a, b; Schlotzer-Schrehardt et al. 2005). The clarification of the role of adenosine and its receptors in cancer development may hold great promise for the chemotherapeutic treatment of patients affected by malignancies (Merighi et al. 2003). It has been suggested that adenosine inhibits tumour cell growth while maintaining bone marrow cell proliferation through the involvement of the A_3 receptors (Fishman et al. 2000). Studies related to a murine model demonstrated that the activation of A_3 receptors can interfere with the tumour cell recognition and with the cytolytic activity of cytotoxic lymphocytes, thus leading to the hypothesis that A_3 antagonists might be useful for the revelation of tumour-associated immunosuppression and facilitate adoptive immunotherapy (Gessi et al. 2004a). Evidence of high levels of expression of A_3 adenosine receptor subtype has been provided in several tumour cell lines and A_3 specific antagonists seem to synergistically enhance cytotoxic treatment and counter P-glycoprotein efflux in multi-drug resistance (Ohana et al. 2001; Gessi et al. 2001; Merighi et al. 2001). A recent study provides the first evidence that A_3 AR plays a role in colon tumorigenesis and, more importantly, can

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potentially be used as a diagnostic marker or a therapeutic target for colon cancer. (Gessi et al. 2004a).

A promising strategy for the creation of novel therapeutics for the treatment of asthma originated from the studies oriented to the establishment of the precise role of adenosine in the pathogenesis of asthma and the development of appropriate subtype selective agonists/antagonists. A_3 AR receptor stimulation has been demonstrated to mediate inhibitory effects on eosinophils since it also elevates cAMP. However, some experimental reports suggest that A_3 AR antagonists mediate anti-inflammatory effects, thus the rationale for A_3 AR receptor ligands as therapeutic agents remains to be determined (Brown et al. 2008).

Furthermore, A_3 receptor antagonists may be useful in the treatment of glaucoma (Okamura et al. 2004a; Schlotzer-Schrehardt et al. 2005; Avila et al. 2002).

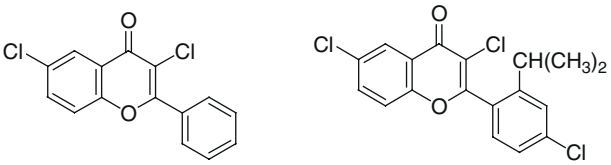
7.2 A_3 AR Antagonists

Initial attempts for obtaining potent and highly selective A_3 AR antagonists focused on a wide pharmacological screening of different heterocyclic compounds. Some xanthine or purine analogues have been firstly examined but none of the tested compounds showed significant affinity or selectivity at rat A_3 adenosine receptor (Kim et al. 1994; Siddiqi et al. 1995; Jacobson et al. 1995). During subsequent evaluations, different classes of non-xanthine nitrogen-containing molecules were identified as potent A_3 antagonists (Müller et al. 2003). In this section, history and perspectives in this field have been summarized with particular notice to the most important reports of the last 5 years.

7.2.1 Non-purine Heterocycles

7.2.1.1 Flavonoid Derivatives

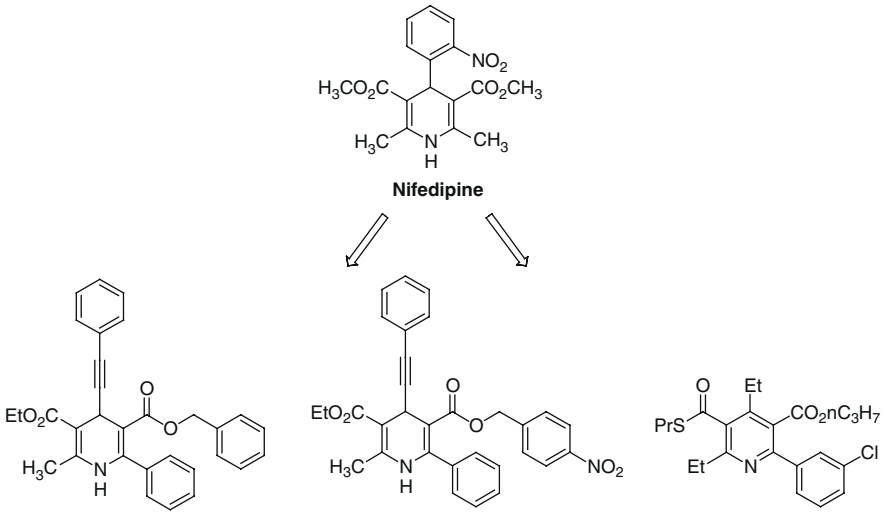
Some flavonoid structures revealed micromolar affinity at human A_3 (hA_3) adenosine receptors after binding screening of different phytochemicals (Ji et al. 1996). The optimisation of flavone nucleus led to the identification of **MRS 1088** and **MRS 1067** (Karton et al. 1996) as the most potent and selective compounds of this series at hA_3 adenosine receptor subtype (Fig. 7.1). CoMFA analysis (Moro et al. 1998) suggested that bulky groups at the C^2 and C^6 positions and at the ortho position of the phenyl ring increase hA_3 affinity. Another interesting observation regards the affinity at rat A_3 receptors; in fact, none of the tested derivatives showed significant affinity at this receptor with consequent loss of selectivity. This experimental datum proved the existence of dramatic inter-species differences in the A_3 receptor aminoacid sequence (only 74% of homology between rat and human, Olah Me and Stiles 1995).



MRS 1088
A_1 = 15 % (10^{-4} M)
A_{2A} = 54,500 nM
hA_3 =741 nM

MRS 1067
A_1 = 36 % (10^{-4} M)
A_{2A} = 19 % (10^{-4} M)
hA_3 = 561 nM

Fig. 7.1 Flavonoid derivatives as A_3 AR antagonists



Nifedipine

MRS 1191
rA_1 = 40,100 nM
rA_{2A} = <10% (10^{-4} M)
hA_3 = 31.4 nM

MRS 1334
rA_1 = > 100 μM
rA_{2A} = >100 μM
hA_3 = 2.69 nM

MRS 1505
rA_1 = > 41,400 nM
rA_{2A} = >24,100 nM
hA_3 = 7.94 nM

Fig. 7.2 1,4-Dihydropyridines

7.2.1.2 1,4-Dihydropyridines and Pyridines

Widely investigated as modulators of L-type calcium channels, several 1,4-dihydropyridines (e.g., Nifedipine, Fig. 7.2) proved also to bind A_1 adenosine receptor in rat brain (Hu et al. 1987; Ismail et al. 1995). Considering the similarity between A_1 and A_3 subtypes, Jacobson and co-workers exploited the 1,4-dihydropyridine nucleus as a template for probing SAR profile at A_3 adenosine receptor. It was possible to separate the antagonism of L-type calcium channels from adenosine receptor antagonism, meanwhile enhancing A_3 AR selectivity, through the introduction of

6-aryl and either a 4-phenylethynyl or a 4-styryl substitution (Van Rhee et al. 1996). Such analysis indicated that sterically bulky groups are tolerated at 4-, 5- and 6-positions. The replacement of the methyl ester at the 5-position of nifedipine with the larger benzyl ester, combined with the introduction of a phenyl ring and a phenylethynyl moiety at the 6- and 4-positions respectively, led to compound **MRS 1191** (Fig. 7.2) (Jiang et al. 1996, 1997). Also the effect of the stereochemistry at the C_4 centre has been evaluated, demonstrating that the S-isomer of **MRS 1191** shows better affinity (about 35-fold) than the R-isomer (Jiang et al. 1999).

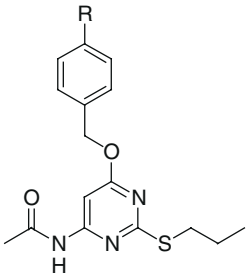
An electron-withdrawing group, such as nitro, at 4′- position of the benzyl ester determined a significant enhancement of both affinity and selectivity (compound **MRS 1334**). Any other modifications, including heterocycles at the 4-position, aminoalkyl or thioalkyl groups at the 3- and 5-positions, appeared detrimental in term of both affinity and selectivity.

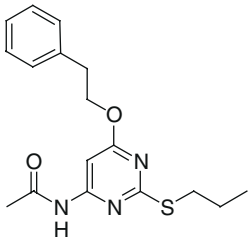
The binding profile at ARs of 3,5-diacyl-2,4-dialkylpyridines obtained from the oxidation of the corresponding 1,4-dihydropyridines has been examined (Van Rhee et al. 1996; Jiang et al. 1996) and the general SAR revealed that the structural requirements for the enhancement of A_3 AR affinity and selectivity don't entirely reflect the outline of the parent dihydropyridines derivatives (Li et al. 1998). The most potent compounds of this series, unlike the dihydropyridines, have been substituted at the 4-position with small alkyl groups (**MRS 1505**, 5-propyl-2,4-diethyl-3-(propylsulfanylcarbonyl)-6-phenyl-pyridine-5-carboxylate). Potent fluorinated along with hydroxylated pyridine derivatives have been also reported (Li et al. 1999). While the fluorination led occasionally to improve affinity, the inclusion of hydroxyl groups failed to increase A_3 AR affinity and selectivity.

An extension of this research has been performed by Jacobson and co-workers (Xie et al. 1999). This study considered a series of N-alkyl-pyridinium salts which showed to be less potent than the pyridine derivatives, nevertheless, the retention of A_3 receptor selectivity and, above all, the water solubility of such compounds, suggested to consider the study for further investigation.

7.2.1.3 2-Mercaptopyrimidines

A series of 4-amino-6-hydroxy-2-mercaptopyrimidine derivatives has been synthesized and biologically evaluated as A_3 AR antagonists (Cosimelli et al. 2008). The design of these molecules originated from the SAR optimization of a triazolopyrimidinone derivative, previously identified using a 3D database search. The stepwise lead optimization resulted in compounds with very potent affinity and selectivity at the hA_3 AR such as **1**–**3** (Fig. 7.3). The best results have been obtained introducing a propylsulfanyl and an acetamino moieties at the 2- and 4-positions, respectively, and a 4-substituted-benzyloxy or a phenethyloxy at the 6-position of the pyrimidine nucleus. The cAMP functional assays for selected molecules showed a behaviour as full antagonists. The most potent compounds at the A_3 AR, (**3**, hA_3 (IC_{50}) = 1.25 nM), was docked into a published model of this receptor and several important attractive interactions have been highlighted. The model binding mode of **3** showed to be consistent with mutagenesis data reported in literature and furnished important information for the design of new hA_3 AR antagonists based on a substituted-pyrimidine nucleus.



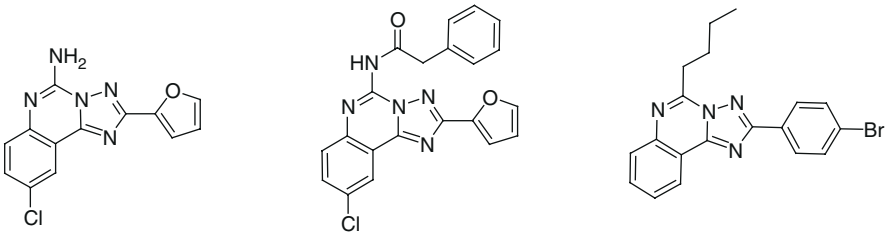


1, R = Cl	**2, R = OCH**	**3**
hA_1 (K_i) > 10,000 nM	hA_1 (K_i) = 2443 nM	hA_1 (K_i) = 2080 nM
hA_{2A} (K_i) > 10,000 nM	hA_{2A} (K_i) = 47% (10 μM)	hA_{2A} (K_i) =27% (10 μM)
hA_{2B} (EC_{50}) > 10,000 nM	hA_{2B} (EC_{50}) > 10,000nM	hA_{2B} (EC_{50}) > 10,000 nM
hA_3 (K_i) = 3.5 nM	hA_3 (K_i) = 1.8 nM	hA_3 (K_i) = 1.4 nM
hA_3 (IC_{50}) = 3.5nM	hA_3 (IC_{50}) = 2.7 nM	hA_3 (IC_{50}) = 1.25 nM

Fig. 7.3 2-Mercaptopyrimidines

7.2.1.4 Triazoloquinazoline

The development of the triazoloquinazoline series derived from the experimental observation that the well-known adenosine antagonist 9-chloro-2-(2-furanyl)-[1,2,4] triazolo[1,5-c]-quinazoline-5-amine (**CGS 15943**, Fig. 7.4) binds in the nanomolar range to the human A_3 receptor with lack of selectivity vs. A_1 A_{2A} and A_{2B} adenosine receptors (Ongini et al. 1999). Jacobson and co-workers (Kim et al. 1996, 1998) demonstrated that the acylation of 5-amino group of **CGS 15943** enhances both affinity and selectivity at the hA_3 adenosine receptors, in particular, compound **MRS 1220** showed subnanomolar affinity at the hA_3 AR with ~80-fold and ~16-fold selectivity vs. A_1 and A_{2A} subtypes respectively. Other modifications of **CGS 15943** structure were evaluated, such as substitutions at the furan and the phenyl rings or replacement of the 5-amino group with a carbonyl function, but none of the synthesised derivatives showed significant affinity and selectivity at hA_3 adenosine receptor. A representative collection of triazoloquinazolines reported by Jacobson (Kim et al. 1998) has been selected as a data set for a QSAR analysis for relating the A_3 adenosine receptor affinity of compounds to their physicochemical properties (Dooil et al. 2006). This report highlights that the electronic and spatial characteristics of substituents in triazoloquinazoline derivatives seem to provide critical contribution to ligand–receptor binding whereas, the hydrophobic properties exert a negligible effect and their importance seems strictly confined to the chlorophenyl moiety of these compounds. The rimoval of the 9-Cl atom along with the replacement of the 5-phenylacetamido and 2-furyl moieties with a linear alkyl chain and a 4-Br-phenyl ring respectively, led Okamura et al. to the discovery of derivative **4** as potent and selective A_3 AR antagonist (Okamura et al. 2004a).

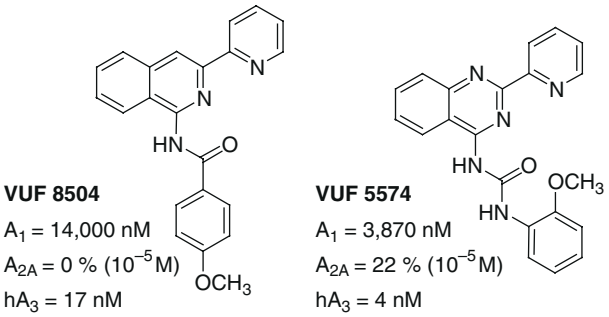


CGS 159434
hA_1 (K_i) = 3.5 nM
hA_{2A} (K_i) = 0.4 nM
hA_{2B} (IC_{50}) = 44 nM
hA_3 (K) = 95 nM

MRS 1220
rA_1 (K_i) = 52.7 nM
rA_{2A} (K_i) = 10.3 nM
hA_3 = 0.65 nM

4
hA_1 (IC_{50}) = > 10000 nM
hA_{2A} (IC_{50}) = > 10000 nM
hA_{2B} (IC) = > 10000 nM
hA_3 (IC_{50}) = 8.2 nM

Fig. 7.4 Triazoloquinazoline derivatives



VUF 8504
A_1 = 14,000 nM
A_{2A} = 0 % (10^{-5}M)
hA_3 = 17 nM

VUF 5574
A_1 = 3,870 nM
A_{2A} = 22 % (10^{-5}M)
hA_3 = 4 nM

Fig. 7.5 Isoquinoline (VUF8504) and quinazoline (VUF 5574) derivatives as A_3 AR antagonists

7.2.1.5 Isoquinolines and Quinazolines

In 1998 Ijzerman and co-workers found that a series of 3-(2-pyridinyl)-isoquinoline derivatives presented A_3 affinity and, in order to improve the binding profile of such compounds, they introduced different substituents at 1- and 3-positions (Van Muijlwijk-Koezen et al. 1998a, 1998b, 2000). The best results in this field were obtained with compound **VUF 8504** (Fig. 7.5) in which the 2-pyridinyl moiety is unsubstituted while at 1-position has been introduced a phenyl ring linked to the quinoline nucleus by an amidic spacer and substituted at 4-osition with an electron donating group such as $–OCH_3$. In the same studies, the effect of an additional nitrogen atom was estimated by synthesising bioisosteric quinazoline derivatives, but it could be asserted that the modification doesn't play an important role on the interaction between the target molecules and hA_3 receptors. An increased affinity in this class of compounds has been obtained by substitution of the amide spacer with an urea moiety. **VUF 5574** (Fig. 7.5) showed affinity at hA_3 AR in the nanomolar range, while ineffective at A_1 and A_{2A} receptor subtypes.

7.2.1.6 Thiazole and Thiadiazole

The bicyclic system of isoquinoline and quinazoline has been replaced by several monocyclic rings (Van Muijlwijk-Koezen et al. 2001). Some thiazole and thiadiazole derivatives revealed to be the most promising candidates for the identification of new A_3 AR ligands.

Derivative *N*-[3-(4-methoxy-phenyl)-[1,2,4]thiadiazol-5-yl]-acetamide (**5**, Fig. 7.6) has been claimed as the most potent A_3 antagonist of the series exhibiting a K_i value of 0.79 nM at hA_3 AR and antagonistic property in a cAMP functional assay (Jung et al. 2004). A series of potent and selective A_3 AR antagonists has been obtained via an optimization study of compound **6** revealing that a 5-(pyridine-4-yl) moiety on the 2-aminothiazole ring was optimal for enhanced receptor potency and selectivity (Press et al. 2004). Of particular note resulted *N*-[4-(3,4,5-trimethoxyphenyl)-5-pyridin-4-ylthiazol-2-yl]-acetamide **7** showing subnanomolar affinity at the human A_3 AR with 1,000-fold selectivity against the other adenosine receptors. This compound confirmed the behaviour as competitive antagonist of I-AB-MECA.

Binding affinity data of thiazoles and thiadiazoles at the hA_3 AR have been subjected to QSAR analysis (Bhattacharya et al. 2005). This study disclosed the importance of molecular electrostatic potential surface (Wang-Ford charges) in correspondence of atoms C2, C5, C7, X8 and S9 (Fig. 7.6), the last two playing the most important roles. Furthermore, the A_3 binding affinity increases with decrease of lipophilicity of the compounds and in the presence of short alkyl chains (Me or Et) at the R position.

The introduction of an aromatic acyl substituent at the 2-position of the thiazole ring, such as benzoyl, nicotinoyl (compound **8**, Fig. 7.6), or isonicotinoyl, led to

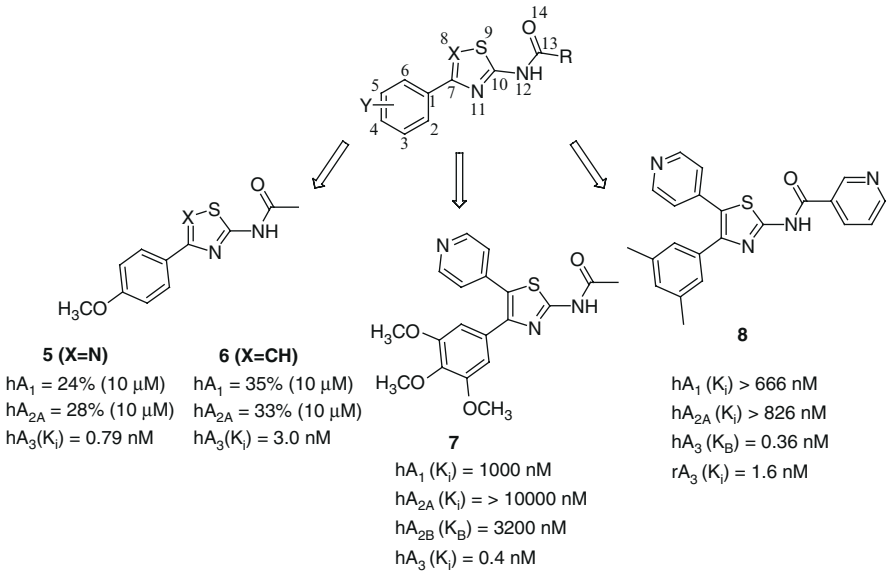


Fig. 7.6 Thiazoles and thiadiazoles

compounds with good human and rat A_3 AR affinity as well as selectivity over hA_1AR and $hA_{2A}AR$. In an in vivo rat model compound **8** inhibited IB-MECA-induced plasma protein extravasation in the skin of rats, demonstrating a good oral absorption profile and bioavailability (Miwatashi et al. 2008). It also increased the anti-asthmatic effect of dexamethasone by combination therapy and these results suggested that the A_3AR antagonist could become a new type of anti-asthma drug as an enhancer of steroids.

7.2.1.7 Pyrazoloquinolines

The binding affinity at bovine A_1 and A_{2A} and at human cloned A_3 adenosine receptors of some 2-arylpyrazolo[3,4-*c*]quinolin-4-ones along with their corresponding 4-amines and 4- substituted-amino derivatives have been reported by Colotta et al. in 2000 (Colotta et al. 2000). The 4-benzoylamido derivative **9** (Fig. 7.7) exploited one of the best binding profile of the series as A_3 AR antagonist. The same group recently reported an extension of the SAR study about this class of compounds (Colotta et al. 2007) highlighting that bulky and lipophilic acyl-amino groups at the 4-position seemed able to promote hA_3 AR potency and selectivity.

Selected compounds of these series were tested in an in vitro rat model of cerebral ischemia and proved to prevent the irreversible failure of synaptic activity induced by oxygen and glucose deficiency in the hippocampus, thus confirming that potent and selective A_3 AR antagonists may substantially increase the tissue resistance to ischemic damage.

The synthesis and the affinity profile at ARs of a series of 2-phenyl-2,5-dihydro-pyrazolo[4,3-c]quinolin-4-ones, conceived as structural isomers of the parent 2-arylpyrazolo[3,4-c]quinoline derivatives, have been as well reported (Baraldi et al. 2005a). Some of the synthesized compounds showed A_3 AR affinity in the nanomolar range and good selectivity as evaluated in radioligand binding assays at hARs. In particular, substitution at the 4-position of the 2-phenyl ring by methyl, methoxy, or chlorine and the presence of a 4-oxo functionality gave good activity and selectivity (compound **10**).

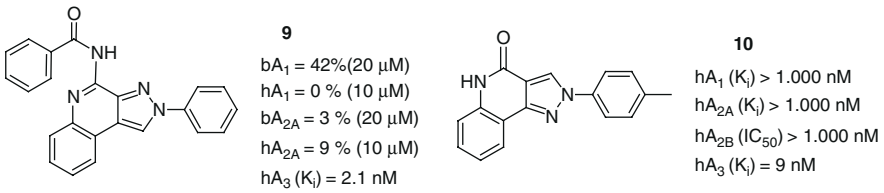


Fig. 7.7 2-Arylpyrazolo[3,4-c]quinoline (9) and 2-aryl-pyrazolo[4,3-*c*]quinoline (10) derivatives

7.2.1.8 Triazoloquinoxalines

Triazolo[4,3-a]quinoxalines

Interesting studies performed in the last decade by Colotta and co-workers highlighted that the 1,2,4-triazolo[4,3-*a*]quinoxalin-1-one moiety is an attractive scaffold for obtaining potent and selective hA_3 AR antagonists (Colotta et al. 2004; Lenzi et al. 2006). An intensive synthetic work based on the systematic substitution of the 2-, 4- and 6-positions of the tryciclic template along with molecular modeling investigations performed to rationalize the experimental SAR findings, led to the identification of the optimal structural requirements for A_3 AR affinity and selectivity. In particular the introduction in the triazoloquinoxaline moiety of a 4-oxo (compound **11**) or 4-*N*-amido (compound **12**, Fig. 7.8) functions affords selective and/or potent A_3 AR antagonists indicating that a C=O group, either extranuclear or nuclear, is necessary for A_3 affinity. This suggested the probable engagement of this site of the molecule in a hydrogen bond with the A_3 AR binding site. Hindering and lipophilic 4-acyl-amino moieties showed to enhance A_3 AR affinity (compound **12**). Substitution of the 4′-position of the 2-phenyl ring with a methoxy or a nitro groups and the 6-nitro substitution as well the combination of these substituents afforded nanomolar A_3 AR affinity and better A_3 selectivity. 1-Oxo, 6-nitro, and 4-amino groups have been supposed to be involved in hydrogen bonds anchorage to the binding site.

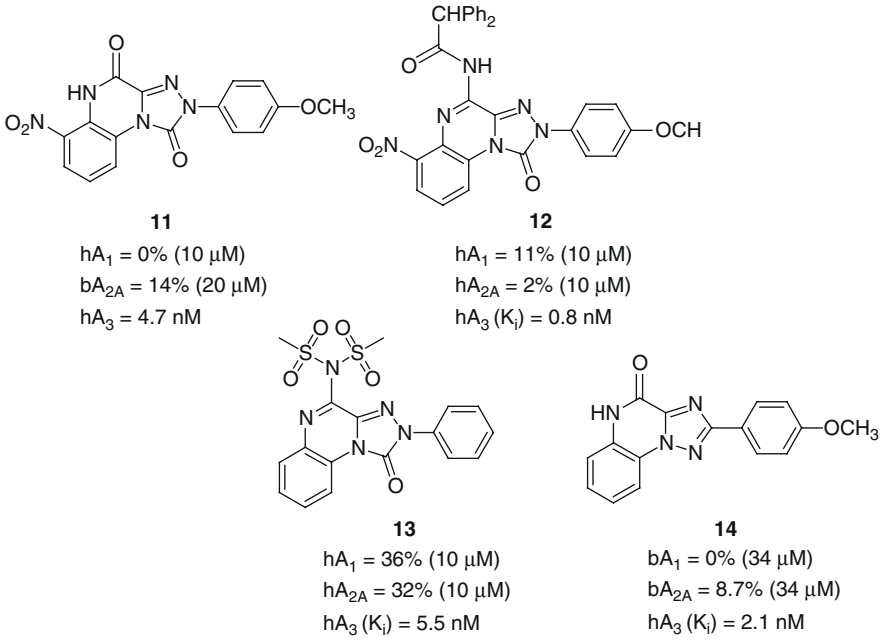


Fig. 7.8 Triazolo[4,3-*a*]quinoxalines (11-13) and triazolo[1,5-*a*]quinoxalines (14)

The selected 4-bismethanesulfonylamino-2-phenyl-1,2,4-triazolo[4,3-a] quinoxalin-1-one, which shows high hA_3 affinity (**13**, K_i = 5.5 nM) and selectivity versus hA_1, hA_{2A} (both selectivity ratios > 1,800) and hA_{2B} (cAMP assay, IC_{50} > 10,000 nM) receptors, recently showed protective effect on cerebral ischemia induced by oxygen and glucose deprivation (Colotta et al. 2008).

Triazolo[1,5-a]quinoxalines

Some 2-aryl-8-chloro-1,2,4-triazolo[1,5-a]quinoxaline derivatives, have been synthesized and tested in radioligand binding assays at bA_1 and bA_{2A} and at hA_1 and hA_3 AR (Catarzi et al. 2005a, b). The SAR of these compounds are in agreement with those of previously reported 2-aryl-1,2,4-triazolo[4,3-*a*]quinoxalines and 2-arylpyrazolo[3,4/4,3-c]quinolines, thus suggesting a similar AR binding mode. This studies provided some interesting compounds and among them the 2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-a]quinoxalin-4-one (**14**), which is the most potent and selective hA_3 AR antagonist of this series.

7.2.1.9 Pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines

The first example of adenosine receptor antagonist, containing the pyrazolo-triazolo-pyrimidine scaffold (Baraldi et al. 2002a) was reported by Gatta and co-workers (Gatta et al. 1993).

A wide number of compounds (MRE series) originated from the structure-activity optimization work based on the systematic substitution at the C^2, C^5, C^9, N^7, N^8positions. (Baraldi et al. 2006; Bolcato et al. 2008; Cacciari et al. 2007).

The N^7 substituted derivatives revealed to have mainly affinity for the hA_{2A} AR (Baraldi et al. 2002b) while the most potent and selective compounds at the hA_3 AR subtype derived from the combination of a small alkyl chain at the N^8 pyrazole position with a (substituted)phenylcarbamoyl chain at the N^5-position (Baraldi et al. 2003). Compound labelled **MRE-3008-F20** (Fig. 7.9), one of several high-affinity antagonists of this series, is an hA_3 AR ligand (K_i = 0.29 nM against [^{125}I]-AB-MECA binding to human receptors expressed in HEK293 cells) with good selectivity over the other hARs. It showed antagonist activity in a functional assay being capable to block the effect of IB-MECA on cAMP production in CHO cells with an IC_{50} value of 4.5 nM. **[^{3}H]-MRE 3008-F20** shows a K_D value of 0.82 ± 0.08 nM and Bmax value of 297 ± 28 fmol/mg protein (Varani et al. 2000).

A relevant problem of pyrazolo-triazolo-pyrimidines was the typical low water solubility, which limited their employment as pharmacological and diagnostic tools. The bioisosteric replacement of the phenyl ring of the 5-phenylcarbamoyl moiety with a 4-pyridyl moiety (Maconi et al. 2002) provided high water solubility while enhancing hA_3 AR affinity. Compound **MRE-3005-F20** and the corresponding HCl salt, showing very high affinity and good selectivity at hA_3 receptor subtype with K_i values in the picomolar range (40 and 10 pM respectively), can be considered

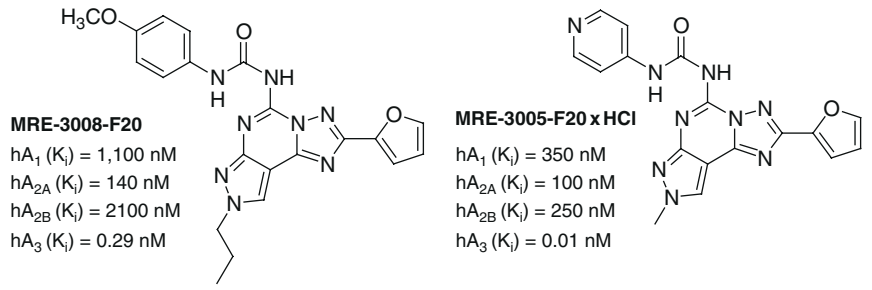


Fig. 7.9 Pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines

ideal candidates for pharmacological and clinical investigation of the hA_3 AR subtype. Receptor modelling ascribed this increase of affinity, compared to neutral arylcarbamate derivatives, to strong electrostatic interactions between the pyridinium moiety and the side chain carbonyl oxygen atoms of Asn274 and Asn278, both located on TM7. Additional studies suggested the involvement of a residue Tyr254 in an H-bond with the pyridyl ring able to justfy both enhancement of receptor affinity and selectivity (Tafi et al. 2006). The replacement of the N^5-pyridine moiety with several N^5-heteroaryl rings produced a general loss of affinity and selectivity at the hA_3 AR (Giorgia Pastorin et al. 2006)

To rationally design and synthesize hA_3 AR antagonists with improved binding and/or ADME profiles and "drugability" different molecular modeling investigations have been carried out in the last years with a particular notice to the pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines family as the most potent class of A_3 AR antagonists ever reported (Tafi et al. 2006). A combined target-based (high throughput molecular docking) and ligand-based (CoMFA) drug design approach has been recently performed by Moro and co-workers (Moro et al. 2005) which defined a novel "Y-shaped" binding motif for pyrazolo-triazolo-pyrimidines and rationally delineated some key ligand–receptor interactions for this class of molecules: (1) A steric control around the 3- and 4-positions of the N^5-phenyl ring justifies the decrease of affinity of 3- or 4-substituted-phenyl derivatives; (2) an important π-π interaction takes place between the 2-furyl ring and two phenylalanine residues of the binding site; (3) an hydrophobic pocket, bordered by two hydrophilic amino acids, surrounds the N^8 interaction area; (4) strong H-bonding is possible between a residue of Asn and the N^4 of the triazolo ring.

7.2.1.10 Various Heterocycles

Other classes of heterocyclic compounds have been identified as A_3 adenosine receptor antagonists, but none of these could be classified into particular family groups due to the high structural differences.

Compound **15** (Fig. 7.10) deserves to be mentioned in the preset report not just for its good binding profile as A_3 AR antagonists but especially for the novelty of

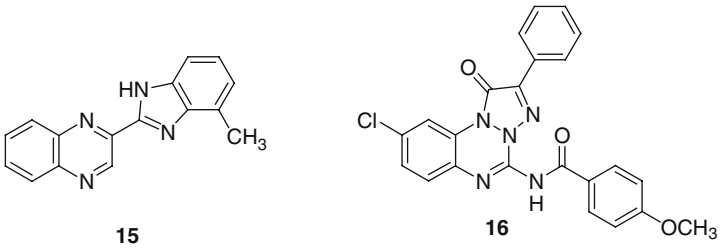


Fig. 7.10 Various heterocyclic compounds as A_3 AR antagonists

the strategy applied for its design based in a 3D database searching approach (Novellino et al. 2005). There are increasing evidences of the relevance of 2D/3D database searching as a valuable tool to discover novel lead compounds.

The structural manipulation of a series of phenyltriazolobenzotriazindiones, previously described as ligands at the central benzodiazepine receptor, led Da Settimo and co-workers to the identification of a series of aminophenyltriazolobenzotriazinones among which compound **16**, as a result of a systematic lead optimization, stands out for its remarkable potency and selectivity at the A_3 AR (K_i values at the A_1, A_{2A}, A_3 ARs of 2,700, >10,000, 1.6 nM respectively and IC_{50} value from cAMP assay at the A_{2B} > 1,000 nM) (Da Settimo et al. 2007). Interestingly, the triazolobenzotriazinone nucleus presents isomeric analogy with the above described triazoloquinoxalinone series (compounds **11–13**).

7.2.2 Purine Derivatives

7.2.2.1 Adenines

The first class of A_3 AR selective antagonists with a bicyclic structure strictly correlated to the adenine nucleus has been claimed in 2005 by Biagi et al. (2005). The authors described the synthesis of a series of N^6-ureidosubstituted-2-phenyl-9-benzyl-8-azaadenines whose adenine-like structure was responsible of the antagonist activity and whose phenylcarbamoyl group ensure selectivity at the A_3 AR. The structure–activity relationship studies was performed basing on the systematic optmization of substituents at the 2-, 6- and 9-positions of the bycyclic scaffold and guided to the desired enhancement of A_1/A_3 selectivity (compound **17**, Fig. 7.11).

Basing on the finding that the known differentiation agent "reversine" (2-(4-morpholinoanilino)-N^6-cyclohexyladenine) exerted a moderate antagonist activity at the hA_3 AR (K_i value of 0.66 μM), Jacobson and co-workers developed a series of reversine analogues focusing the attention on the substitution pattern at

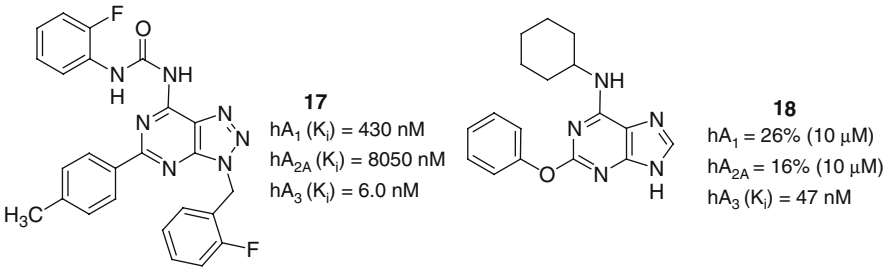


Fig. 7.11 Adenines and 8-azaadenines

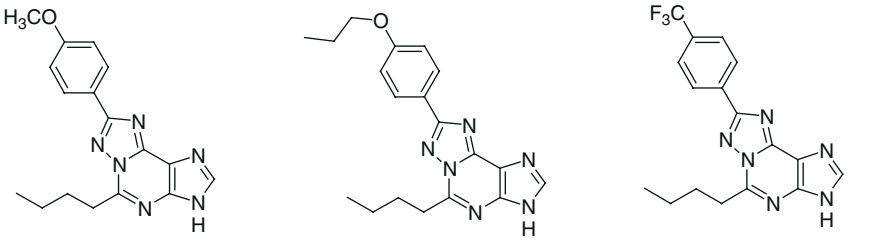


Fig. 7.12 Triazolo[5,1-*i*]purines

the 2- and N^6-position of the adenine scaffold (Perreira et al. 2005). One of most interesting compound in terms of hA_3 AR affinity and selectivity combines the N^6-cyclohexyl moiety of reversine with a 2-phenyloxy group (compound **18**). A few derivatives tested in binding assays to the rat A_3 AR seemed to reflect the species dependence of the affinity typical of most known A_3 AR antagonists, showing to be inactive at 10 μM.

7.2.2.2 Triazolopurines

Okamura et al. (2002, 2004a) recently reported the study of a new series of 1,2,4-triazolo[5,1-i]purines. This research group highlighted the structural similarity between the new class of compounds and the triazoloquinazoline derivatives and consequently evaluated the corresponding A_3 adenosine receptor affinities. These investigations led to potent and selective hA_3 ligands, the most potent of which are reported in Fig. 7.12 (**19, 20**), in particular 5-*n*-butyl-8-(4-*n*-propoxyphenyl)-3*H*-[1,2,4]triazolo[5,1-i]purine **20** exhibited the best selectivity profile of this series

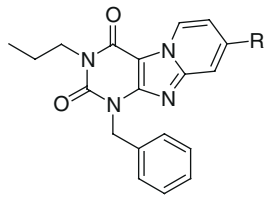
(affinity ratios vs other receptor subtypes >19,600). Compound **OT-7999** demonstrated to significantly reduce intraocular pressure in cynomolgus monkeys at 2–4 h following topical application (500 mcg) (Okamura et al. 2004b).

7.2.2.3 Tricyclic Xanthines

Natural antagonists for ARs, such as caffeine and theophylline show in general low affinity for the A_3 AR subtype (Baraldi et al. 2003). In a recent work, the approach based on the annelation of xanthine derivatives for the development of adenosine receptors antagonists has been extensively considered (Drabczyńska et al. 2003).

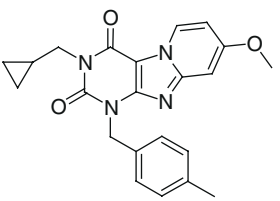
Some pyrido[2,1-*f*]purine-2,4-dione derivatives, which could be considered as tricyclic xanthine derivatives, have been reported to exert low nanomolar affinity at hA_3 (Priego et al. 2002). The most potent compound of this recent series is the 1-benzyl-3-propyl-1*H*,3*H*-pyrido[2,1-f]purine-2,4-dione derivative (**21,** Fig. 7.13) which presents a K_i value of 4.0 ± 0.3 nM at hA_3. The replacement of the benzyl nucleus at 1- position with a methyl moiety determined a dramatic loss of both affinity and selectivity. A new series of 1H,3H-pyrido [2,1-f]purine-2,4-diones carrying a methoxy group at the 8-position, a cyclopropylmethyl group at the N^3-and substituted benzyl groups the 1-positions, revealed a general maintenance of hA_3 AR affinity and a relevant enhancement of the overall selectivity (compounds **23** showed the best binding profile of the series, Priego et al. 2008).

The effect of the replacement of the pyridine ring of the pyrido[2,1-*f*]purine-2,4-dione core with different 5-membered heterocycles has been examined. In particular, the synthesis and the SAR profile at the adenosine receptors of a series of 1-benzyl-3-propyl-7-aryl/alkyl-1*H*,6*H*-pyrrolo[2,1-*f*]purine-2,4-dione and 1-benzyl-3-propyl-7-aryl/alkyl-1*H*,8*H*-imidazo[2,1-*f*]purine-2,4-dione derivatives have been recently reported (Baraldi et al. 2005b). Among the examined tricycles, the imidazo[2,1-*f*]purine-2,4-dione derivatives were 2- to 10-fold more potent than the corresponding pyrrolo[2,1-*f*]purine-2,4-dione derivatives. The best results were obtained with the introduction of small alkyl chains at the 7 position (1-benzyl-7-methyl-3-propyl-1*H*,8*H*-imidazo[2,1-*f*]purine-2,4-dione **24,** 1-benzyl-7-ethyl-3-propyl-1*H*,6*H*-pyrrolo[2,1-*f*]purine-2,4-dione **25**). Compound **24** shows a subnanomolar affinity towards A_3 adenosine receptor target with a noteworthy selectivity versus the other adenosine receptors subtypes (K_i (hA_3) = 0.8 nM, K_i (hA_1/hA_3) = 3,163, K_i (hA_{2A}/hA_3) > 6,250, IC_{50} (hA_{2B})/K_i (hA_3) = 2,570). A SAR extension of this project has been recently realized performing new substitutions alternatively at the 1-, 3- and 8-positions of the reference compound **24** (Baraldi et al. 2008). Thanks to the identification of versatile synthetic route the imidazo[2,1-*f*]purin-2,4-dione nucleus has been manipulated at various positions and a SAR analysis combined with a molecular modeling study originated from this work. Some of the new reported compounds confirmed high A_3 AR binding affinity in association with relevant selectivity versus the remaining ARs (1-benzyl-7-methyl-3-allyl-1*H*,8*H*-imidazo[2,1-*f*]purine-2,4-dione**,** K_i hA_3 = 5.13 nM, hA_1-hA_{2A}-hA_{2B}/hA_3 >195; 1-benzyl-7-methyl-3-prop-2-ynyl-1*H*,8*H*-imidazo[2,1-*f*]purine-2,4-dione K_i hA_3 = 15 nM, hA_1-hA_{2A}-hA_{2B}/hA_3 > 67). Compounds 8-bromo-7-(2-oxo-propyl)-1-propyl-3-pyridin-2-ylmethyl-3,7-dihydro-

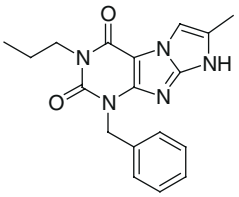


R = H, 21
hA_1 (K_i) = 50 nM
hA_{2A} (K_i) = 119 nM
hA_3 (K_i) = 4.0 nM

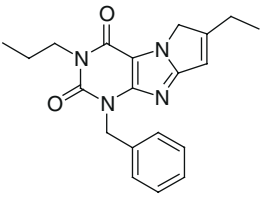
R = Ph, 22
hA_1 = 25% (10 μM)
hA_{2A} = 8% (10 μM)
hA_3 (K_i) = 35 nM



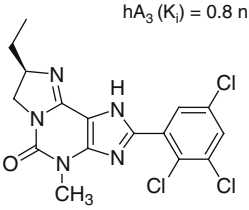
R = H, 23
hA_1 (K_i) = 24% (1 μM)
hA_{2A} (K_i) = 0% (1 μM)
hA_3 (K_i) = 2.2 nM



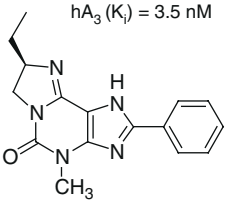
24
hA_1 (K_i) > 1000 nM
hA_{2A} (K_i) > 1000 nM
hA_{2B} (IC_{50}) > 1000 nM
hA_3 (K_i) = 0.8 nM



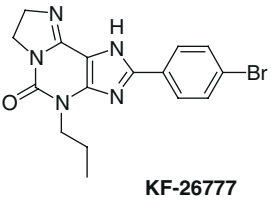
25
hA_1 (K_i) > 1000 nM
hA_{2A} (K_i) > 1000 nM
hA_{2B} (IC_{50}) > 1000 nM
hA_3 (K_i) = 3.5 nM



PSB10
rA_1 (K_i) = 805 nM
hA_1 (K_i) = 1700 nM
rA_{2A} (K_i) = 6040 nM
hA_{2A} (K_i) = 2,700 nM
hA_3 (K_i) = 0.43 nM



PSB11
rA_1 (K_i) = 440 nM
hA_1 (K_i) = 1,640 nM
rA_{2A} (K_i) = 2,100 nM
hA_{2A} (K_i) = 1,280 nM
hA_3 (K_i) = 2.3 nM



KF-26777
hA_1 (K_i) = 1800 nM
hA_{2A} (K_i) = 470 nM
hA_{2B} (K_i) = 620 nM
hA_3 (K_i) = 0.2 nM

Fig. 7.13 Pyrido/imidazo/pyrrolo-purinones as tricyclic xanthines with high affinity and potency at the hA_3 AR subtype

purine-2,6-dione hydrochloride (K_i hA_3 = 34 nM) and 1-benzyl-7-methyl-3-(2-hydroxy-ethyl)-1*H*,8*H*-imidazo[2,1-*f*]purine-2,4-dione (K_i hA_3 = 38 nM), although less potent than the reference compound, showed a good binding profile in association with enhanced hydrophilic properties on the basis of estimated CLogP values. The binding disposition of these molecules was investigated by means of a docking approach using the mixed pharmacophoric-molecular modeling procedure and the A_3 receptor model previously reported. The obtained results highlighted the interaction of these compounds with the main residues suggested important by mutagenesis studies.

Furthermore the docking poses were used as an alignment tool for the generation of a 3D-QSAR model which was able to quantitative explain the different affinity of the ligands.

The synthesis and the biological evaluation of an analogue series of fused xanthine derivatives, have been investigated by Müller and co-workers (Müller et al. 2002a). In particular the (R)-4-methyl-8-ethyl-2-phenyl-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-i] purin-5-one (**PSB11**) exhibited a K_i value of 2.3 nM for A_3 receptor and good selectivity vs. all other adenosine receptor subtypes. The radiolabelled derivative of this compound ([^{3}H]PSB-11) exhibited a K_D value of 4.9 nM and a Bmax value of 3,500 fmol/mg of protein (Müller et al. 2002b). An important innovation of such series, in comparison with xanthines, is a significant increase of water solubility due to the introduction of a basic nitrogen atom which can be protonated in physiological conditions. Compound **PSB-10** bearing a 2,3,5-trichlorophenyl moiety at the 2-position, showed inverse agonist activity in binding studies in CHO cells expressing recombinant hA_3 ARs (IC_{50}= 4 nM) (Ozola et al. 2003). The 2-(4-bromophenyl)-derivative named **KF-26777** with subnanomolar affinity at the hA_3AR (K_i = 0.2 nM) and high selectivity over A_1, A_{2A} and A_{2B} ARs (9,000-, 23,500-, 31,000-fold respectively), was indicated of potential interest for the treatment of brain ischemia and inflammatory diseases such as asthma (Saki et al. 2002).

7.2.3 Nucleoside-Derived A_3 AR Antagonists

The attempts of the scientific community focused on the design of A_3 AR antagonists with nucleoside structure is aroused by a general limitation characterizing non-purine heterocyclic antagonists the most of which, although having high affinity and selectivity for human A_3 AR, show low or none affinity toward rat A_3 AR. This hampers the drug development processes considering the difficulty to employ animal models for drug testing (Gao et al. 2003).

The structural manipulation at different positions of adenosine provided a wide number of ligands at the A_3 AR exerting different levels of potency, selectivity, and intrinsic efficacy. The exact combination of modifications showed to affect the balance between full agonism, partial agonism and antagonism (Joshi and Jacobson 2005).

A series of 8-alkynyladenosines reported by Volpini et al. (2001) represents the first example of structural adenosine analogues, with the intact ribose moiety, which behave as selective antagonists at hA_3 adenosine receptor. The antagonistic effect was provided by the introduction of different alkynyl chains at the C-8 position of the purine nucleus. Compound **26** (Fig. 7.14) showed high level of selectivity at hA_3 versus all other receptor subtypes, albeit showing low affinity in comparison with other heterocyclic molecules previously described.

It has been demonstrated that the introduction of a benzyl or a 3-iodobenzyl moiety at the N^6- position and a Cl substituent at the 2-position of adenosine synergistically contribute to reduce the intrinsic efficacy of the corresponding nucleoside derivatives.

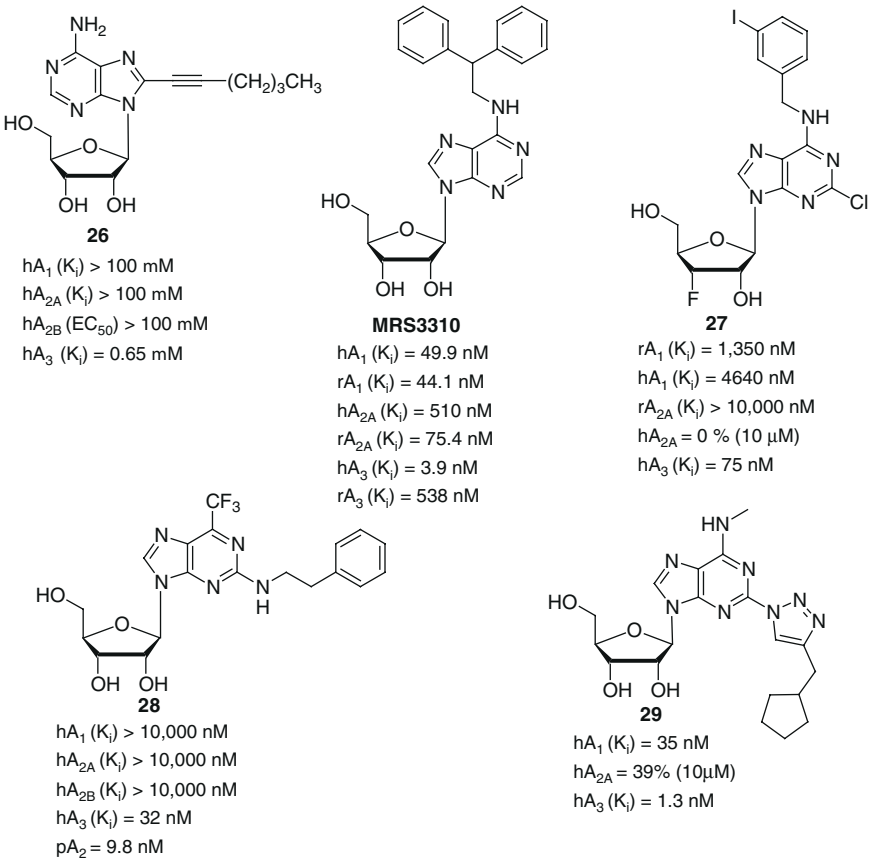


Fig. 7.14 Examples of nucleoside-derived A_3 AR antagonists

Thus N^6-(3-iodobenzyl)adenosine (MRS541, structure not shown) acts as partial agonist (46% A_3 efficacy) while the 2-chloro-N^6-(3-iodobenzyl)adenosine (MRS542, structure not shown) behaves as potent but non selective A_3 AR antagonists (K_i A_3 = 1.8 nM, EC_{50} A_{2B} > 10,000 nM, K_i A_{2A} = 197 nM, K_i A_1 = 16.8 nM) (Gao et al. 2006). It has to be remarked that the efficacy of the latter compound is completely restored by the replacement of the 4′-hydroxymethylene group with a 5′-methyluronamide function (Cl-IB-MECA).

N^6-Substitution of adenosine proved to be determining in affecting intrinsic efficacy considering that adenosine showed to reverse its agonistic activity into antagonism when a N^6-2,2-diphenylethyl moiety has been introduced (**MRS3310**, Fig. 7.14, Tchilibon et al. 2004, 2005).

Some 2′ and 3′-fluoro analogues of Cl-IB-MECA have been investigated as A_3 AR ligands (Lim et al. 2003). While the introduction of a fluorine atom at the 2′-position compromised both A_3 AR binding and activation, the 3′-fluoro substitution generally resulted in partial agonism. Compound **27** is one of the few ligands

of the series in which the presence of the fluorine at the 3′-position led to a total loss of hA_3 efficacy.

A patent by Solvay (Koch et al. 2006) claimed the possibility to treat allergic diseases with a series of new nucleoside derivatives as high affinity A_3 AR antagonists. One of the most important compound has been reported in Fig. 7.14 (compound **28**). This molecule show low nanomolar affinity for A_3AR with high selectivity over the others ARs subtypes and its potent antagonistic activity has been assessed in functional models (pA_2 = 9.8). This findings highlight the importance of substitutions at the 2- and 6-positions of adenosine for receptor activation. In particular these molecules are the only example of A_3 AR antagonists structurally related to adenosine in which the 6-amino group was completely removed, replaced by a trifluoromethyl moiety.

Among the N^6-C^2-disubstituted adenosine derivatives reported in literature, compound 2-(4-cyclopentylmethyl-1,2,3-triazol-1-yl)-N^6-methyl-adenosine **29** displayed hA_3 AR antagonism with 260-fold binding selectivity versus the hA_1 AR corroborating the assessment suggesting that hindered groups at the 2-position promote A_3 AR binding preventing meanwhile its activation (Cosyn et al. 2006).

Researchers by *GlaxoSmithKline* recently examined the pharmacological properties of the novel ligand, (2R,3R,4S,5R)-2-(6-amino-2-{[(1S)-2-hydroxy-1-(phenylmethyl)ethyl]amino}-9H-purin-9-yl)-5-(2-ethyl-2H-tetrazol-5-yl)tetrahydro-3,4-furandiol (**30**, Fig. 7.15), performing binding and functional studies at the human adenosine receptors system (Bevan et al. 2007). Compound **30** displays high affinity in binding assays for the adenosine A_{2A} and A_3 receptors (K_i = 15.8 nM) but the functional assays revealed an opposite response to ligand interaction with the two receptor subtypes. It indeed behaves as a potent agonist at A_{2A} receptor (pEC_{50} 9.0 ± 0.2) acting instead as a competitive antagonist at A_3 AR (pA_2 8.3 ± 0.04).

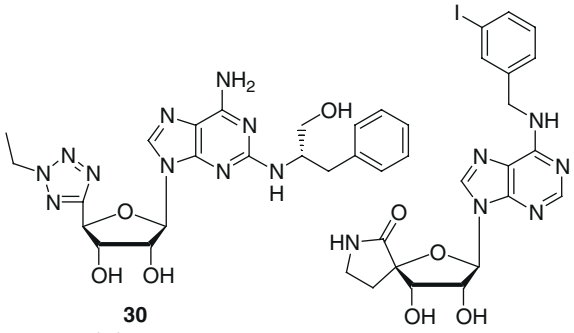


Fig. 7.15 Modifications of the 5′-uronamide moiety of known ARs agonists efficacious in promoting antagonistic behaviour

This ligand has a lower affinity for adenosine A_1 and A_{2B} receptors with pK_i values ≤6. A potent inhibitory effects on the generation of reactive oxygen species from human neutrophils and eosinophils and on the degranulation of human granulocytes subsequent to treatment with compound **30** have been described. This findings provide a useful tool for the understanding of the involvement of A_{2A} and A_3 ARs in inflammation processes.

Compound **MRS1292** (Fig. 7.15), containing a spirolactam moiety, behaves as A_3 adenosine receptor antagonist with K_i value from binding assays of 29.3 nM (Gao et al. 2002). The presence of the additional ring determined the introduction of a conformational restriction which could affect the capacity of the 5′-uronamide to realize an hydrogen bond required for the receptor activation (Gao et al. 2006). **MRS1292** showed to inhibit A_3AR-mediated shrinkage of human nonpigmented ciliary epithelial cells and reduce mouse intraocular pressure, consistent with its putative action as a cross-species A_3 antagonist thus confirming the possible employment of A_3 AR antagonists in the treatment of glaucoma (Yang et al. 2005).

The influence of the ribose ring conformation on binding affinity and activation of the A_3 AR has also been explored using ligands with conformationally restricted ribose rings. Some conformationally constrained analogues of the known A_3 AR agonists IB-MECA have been recently synthesised and tested for their binding affinity as well as for their agonist and/or antagonist activity for ARs (Ravn et al. 2007). Among these series of 2′-*O*,4′-*C*-methylene-β-d-ribofuranosyl nucleosides (locked nucleic acid, LNA nucleosides) and 2'-amino-LNA nucleosides derivatives containing the 2-oxa-5-azabicyclo[2.2.1]heptane scaffold, some potent A_3 AR antagonists have been identified (compounds **31–32**, Fig. 7.16).

The fundamental role of flexibility and H-bonding ability of the 4′-hydroxymethylene or 5′-uronamide moieties of nucleosides derivatives for full activation of the A_3 AR has been lately confirmed by molecular modeling studies (Kim et al. 2006) and the identification of D-4′-thioadenosine derivatives as new nucleoside template for human A_3 AR selective antagonism (Jeong et al. 2007). The highly selective A_3 AR agonists Cl-IB-MECA and its 4′-thio analogue, were successfully

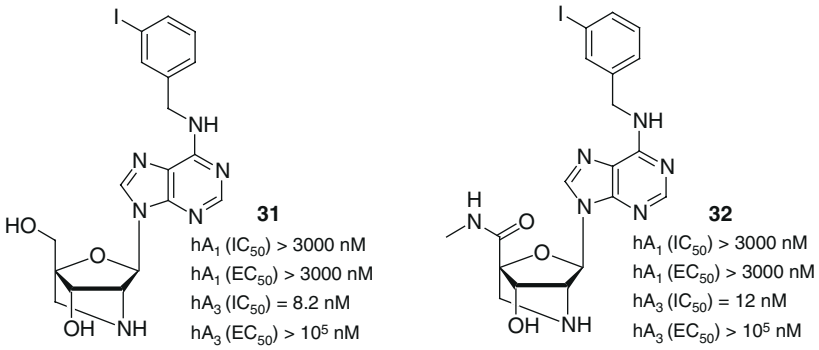
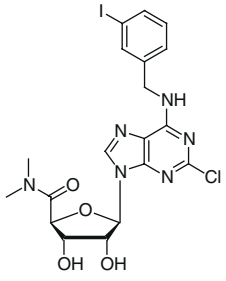


Fig. 7.16 LAN (Locked nucleic acid) nucleosides as conformationally constrained nucleoside-derived A_3 AR antagonists

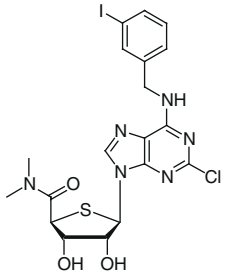
converted into selective antagonists simply by adding a second *N*-methyl group on the 5′-uronamide position (compounds **MRS3771** and **LJ-1256** respectively, Fig. 7.17). Competitive antagonism was demonstrated by Schild analysis (Gao et al. 2006). 5′-*N,N*-Dimethyluronamide derivatives exhibited higher binding affinity than larger 5′-*N,N*-dialkyl or 5′-*N,N*-cycloalkylamide derivatives, indicating that steric factors are crucial in binding to the human A_3 AR (Jeong et al. 2008a).

By completely removing the 4′-substituent of the thionucleoside skeleton of compound **LJ-1256** and optimizing the N^6-substituent, Jacobson and co-workers identified a series of very potent and selective A_3 adenosine receptor antagonists the most effective of which have been depicted in Fig. 7.17 (compounds **33** and **LJ-1251**). All synthesized compounds exhibited excellent binding affinity and selectivity at the A_3 AR and confirmed to be full antagonists in a cyclic AMP functional assay at the hA_3 AR. **LJ-1251** demonstrated also high affinity at the rat A_3 AR expressed in CHO cells (K_i = 3.89 ± 1.15 nM), indicating the possibility of evaluation in small animal models for future drug development and was inactive as agonist or antagonist in a cyclic AMP functional assay at the hA_{2B} AR. Truncated D-4′-thioadenosines have been compared with the corresponding L-type nucleosides in order to evaluate a possible



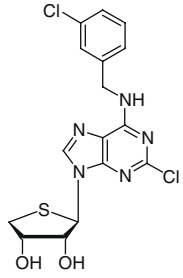
MRS3771

hA_1 (K_i) = 5870 nM
hA_{2A} (K_i) > 10,000 nM
hA_{2B} (EC_{50}) > 10,000 nM
hA_3 (K_i) = 29.0 nM



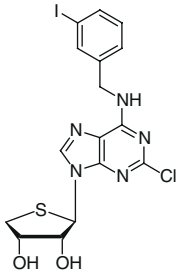
LJ-1256

hA_1 (K_i) = 6220 nM
hA_{2A} (K_i) > 10,000 nM
hA_{2B} (EC_{50}) > 10,000 nM
hA_3 (K_i) = 15.5 nM



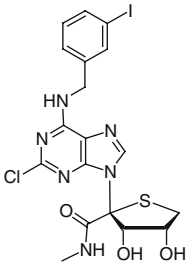
33

hA_1 = 38% (10 μM)
hA_{2A} = 18% (10 μM)
hA_3 (K_i) = 1.66 nM



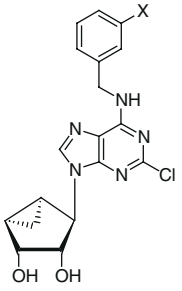
LJ-1251

hA_1 (K_i) = 2490 nM
hA_{2A} (K_i) = 341 nM
hA_3 (K_i) = 4.16 nM



MRS3057

hA_1 (K_i) = 110 nM
hA_{2A} = 47% (10 μM)
hA_3 (K_i) = 4.3 nM



MRS5127, X = I

hA_1 (K_i) = 3040 nM
hA_{2A} (K_i) = 1080 nm
hA_3 (K_i) = 1.44 nM

MRS5147, X = Br

hA_1 (K_i) = 1760 nM
hA_{2A} (K_i) = 1600 nm
hA_3 (K_i) = 0.73 nM

Fig. 7.17 Adenosine and 4′-thioadenosine derivatives as A_3 AR antagonists

stereochemical preference in the binding to the A_3 AR. The L-enantiomers showed to be totally devoid of binding affinity at all subtypes of ARs (Jeong et al. 2008b).

The involvement of the 5′-position of the ribose moiety in receptor activation has been confirmed by recent findings indicating that the shifting of the N^6-(3-iodobenzyl) adenine moiety from the 1′ to the 4′ position of the ribose ring proved to induce A_3 AR potent antagonism in the full agonist 4′-thio analogue of Cl-IB-MECA (see compound **MRS3057**) (Gao et al. 2004). Replacement of the flexible ribose scaffold of prototypical A_3 AR agonists with a bicyclo[3.1.0]hexane ring system resulted in (N)-methanocarba adenosine agonists possessing high potency and selectivity for the A_3 AR subtype. A new series of A_3 AR antagonists belonging to the (N)-methanocarba family has been recently developed thanks to the removal of the N-methylcarboxamide function (see **MRS5127** and **MRS5147**) (Melman et al. 2008).

7.3 Conclusions and Perspectives

A wide number of compounds exerting high potency and selectivity in antagonizing the hA_3 AR has been so far recognized. These molecules are generally characterized by a notable structural diversity taking into account that aromatic nitrogen-containing monocyclic (thiazoles, thiadiazoles, 1,4-dihydropyridines, pyridines, 2-mercaptopyrimidines), bicyclic (flavonoid, isoquinoline, quinozalines, (aza)adenines), tricyclic systems (pyrazoloquinolines, triazoloquinoxalines, pyrazolotriazolopyrimidines, triazolopurines, tricyclic xanthines) and nucleoside-derived antagonists have been identified as potent A_3AR ligands. Probably due to the enigmatic physiological role of A_3AR, whose activation seems related to opposite effects concerning tissues protection in inflammatory and cancer cells, a few molecules have till now reached the preclinical investigation. Indeed the most of the developed A_3AR antagonists is still in biological testing (Integrity source). Among the above described antagonists, compound **OT7999** and its p-chloro congener are expected to enter clinical trials for the treatment of glaucoma while several thiazole derivatives, originated by Novartis, are going to be tested as antiallergic, antiasthmatic and/or antinflammatory drugs. The triazolo quinoline derivative **MRS 1220** reached preclinical screening for the treatment of stroke. Sanofi-Aventis recently led two A_3AR antagonists (SSR-161421 and SAR-137272) into preclinical phase investigation in the therapeutic area of asthma and COPD. **MRS1292** is the only example of nucleoside related A_3 AR antagonists in preclinical phase investigation as antiglaucoma agent.

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Chapter 8
Molecular Modeling and Reengineering of A_3 Adenosine Receptors

Stefano Moro, Erika Morizzo, and Kenneth A. Jacobson

8.1 Introduction

A_3 adenosine receptors (ARs) belong to a small family of GPCRs, which consists of four distinct subtypes, A_1, A_{2A}, A_{2B} and A_3. ARs are ubiquitously expressed in the human body (Fredholm et al. 2001). Many cells express several ARs subtypes, although in different densities. All subtypes, including the A_3 receptor, have been cloned from a variety of species including rat and human (Fredholm et al. 2001). Species differences for A_3 receptors are larger than for other ARs subtypes, particularly between rodent and human (h) receptors (only 74% sequence identity between rat and hA_3 amino acid sequence). This results in different affinities of ligands, particularly antagonists, for rat versus hA_3 receptors. A_3 ARs are negatively coupled to adenylate cyclase via $G_{i2,3}$ (Fredholm et al. 2001; Fishman and Bar-Yehuda 2003). Coupling of the A_3AR to $G_{q/11}$ leading to a stimulation of phospholipase C and its coupling to phospholipase D have also been demonstrated (Parsons et al. 2000). A_3AR stimulation can lead to activation of ERK1/2. In fact, A_3AR agonists stimulate PI3K-dependent phosphorylation of Akt leading to a reduction of the basal levels of ERK1/2 phosphorylation, which in turn inhibits cell proliferation. After exposure to an agonist, A_3ARs undergo rapid desensitization via phosphorylation by G protein-coupled receptor kinase 2 (GRK2) at the intracellular terminal chain (particularly at threonine 318 on the rat receptor) (Palmer and Stiles 2000).

The A_3AR, which is the most recently identified AR, is implicated in a variety of important physiological process (Fishman and Bar-Yehuda 2003). Activation of

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the A_3AR increases the release of inflammatory mediators, such as histamine, from rodent mast cells (Rorke and Holgate 2002), and inhibits the production of tumor necrosis factor-α (TNF-α) (Jacobson 1998). The activation of the A_3AR is also suggested to be involved in immunosuppression and in the response to ischemia of the brain and heart (Stone 2002). It is becoming increasingly apparent that agonists or antagonists of the A_3AR have potential as therapeutic agents for the treatment of ischemic and inflammatory diseases (Linden 2001).

8.2 Homology Modeling of ARs

The development of antagonists, in particular, for the A_3AR has been directed mainly by traditional medicinal chemistry, but the influence of structure-based approaches is increasing (Muller 2003). The evolution of the field of computer-aided design of GPCR ligands, including A_3 agonists and antagonists, has depended on the availability of suitable molecular receptor templates (Becker et al. 2004; Fanelli and De Benedetti 2005; Kristiansen 2004; Moro et al. 2005; Presland 2005). Bovine rhodopsin provided the first high resolution structural information, and for many years, rhodopsin-based homology modeling had been the most widely used approach to obtain three dimensional models of GPCRs. The results of AR modeling based on rhodopsin have been extensively reviewed (Martinelli and Tuccinardi 2008). With the availability of new crystallographic structures it is still questionable which one should be the more appropriate template for GPCRs modeling and, in particular, for ARs.

The percentages of identity of the aligned sequences of the ARs in comparison to GPCRs having an available x-ray crystallographic structure are listed in Table 8.1, and the alignment of the sequences is shown in Fig. 8.1. The percent identity increases from a comparison with bovine rhodopsin to a comparison with hGPCRs. The percent identity is higher if the N-terminus and the C-terminus are not taken into consideration, and the increase is even greater when comparing only TM regions. Naturally, the A_{2A}AR can be considered the best template for homology modeling of the other ARs according to the percent identity of the aligned sequences, but there are some important differences among the ARs that have to be considered in choosing the template for homology modeling. The primary structures of A_1AR, A_{2B}AR, and A_3AR have a similar number of amino acid and, in general, these AR subtypes are among the smaller members of the GPCR family. For example, the human homologs of the A_1AR, A_{2B}AR, and A_3AR consist of 326, 328, and 318 amino acid residues, respectively (Libert et al. 1992; Pierce et al. 1992; Salvatore et al. 1993). In contrast, the hA_{2A}AR consists of 409 amino acids (Furlong et al. 1992), and all cloned species homologs of the A_{2A}AR are of similar mass. This relatively large size is manifested in the carboxyl-terminal tail of the receptor, which is much longer than any of the other AR subtypes.

The TM regions of the GPCRs possess the same overall topology, and the sequence alignment is guided by the most conserved residues in every helix. The size of each helix differs between the crystallographic structures, but the

Table 8.1 Percentage of identity between the aligned sequences of the ARs and those GPCRs for which crystallographic structures are available

		β-Rhodopsin, %	hβ2AR, %	Turkey β1AR, %	$hA_{2A}AR$, %
All	hA_1AR	13.8	19.1	17.2	39.1
	$hA_{2A}AR$	17.8	23.5	22.6	100
	$hA_{2B}AR$	17.8	22.5	20.1	46.6
	hA_3AR	14.1	19.9	17.4	31.3
All except N-term and C-term	hA_1AR	15.6	25.6	24.9	50.8
	$hA_{2A}AR$	20.5	27.9	28.3	100
	$hA_{2B}AR$	22.2	27.9	28.7	61.5
	hA_3AR	15.6	25.6	24.6	41.9
TM regions	hA_1AR	17.7	29.5	31.4	57.7
	$hA_{2A}AR$	22.3	31.8	33.2	100
	$hA_{2B}AR$	22.7	30.5	33.6	69.5
	hA_3AR	17.3	29.5	30.5	49.5
EL2	hA_1AR	14.3	14.8	11.1	32.4
	$hA_{2A}AR$	14.3	11.1	22.2	100
	$hA_{2B}AR$	14.3	18.5	22.2	41.2
	hA_3AR	14.3	11.1	11.1	23.5

loops constitute the most variable region. The second extracellular loop (EL2) is of particular interest for building homology models of GPCRs used for drug design because of its role in the ligand recognition (Moro et al. 1999). The crystallographic structure of the $hA_{2A}AR$ shows a disulfide bond between Cys259 and Cys262 in the intracellular side of the receptor and, in particular, three disulfide linkages that involve the EL2: one between Cys77 and Cys166, that is conserved among the members of family A of GPCRs and connects EL2 and TM3, and two between EL2 and EL1, that are unique to the $A_{2A}AR$ (Cys71-Cys159 and Cys74-Cys146) (Jaakola et al. 2008). The EL2 of the $A_{2A}AR$ defines the extracellular surface properties of the structure and is considerably different from that of rhodopsin. The extensive disulfide bond network forms a rigid, open structure exposing the ligand binding cavity to solvent, possibly allowing free access for small molecule ligands (Jaakola et al. 2008). The turkey β_1 adrenergic receptor and $h\beta_2$ adrenergic receptor structures have the conserved disulfide bridge between EL2 and TM3 (Cys114-Cys189 for β_1AR and Cys106-Cys191 for β_2AR). In addition to this conserved structural constraint, they have a second disulfide bond that involves the EL2 (Cys192-Cys198 for β_1AR and Cys184-Cys190 for β_2AR) (Warne et al. 2008; Rosenbaum et al. 2007; Cherezov et al. 2007). However, rhodopsin has only one cysteine residue in the EL2, which forms a disulfide bond between EL2 and TM3 (Palczewski et al. 2000).

The sequences of the hA_1AR and the hA_3AR contain only one cysteine residue in the EL2 (Cys169 for A_1AR and Cys166 for A_3AR). These residues form the disulfide bridge, common to GPCRs, with the respective cysteine residues of TM3 (Cys80 for A_1AR and Cys83 for A_3AR). The $hA_{2B}AR$ has three cysteine residues in the EL2.

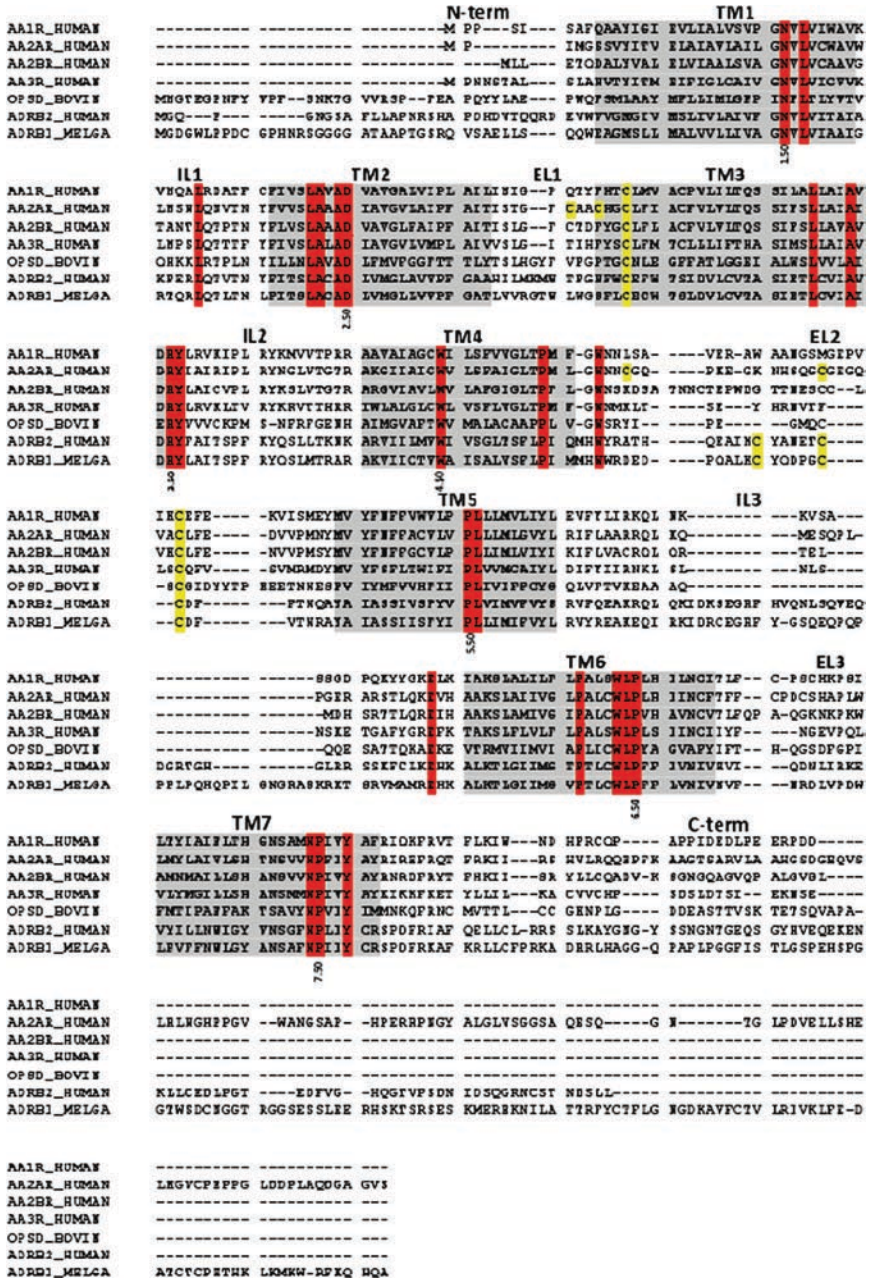

Fig. 8.1 Sequence alignment of the four hARs (A_1, A_{2A}, A_{2B}, A_3), bovine rhodopsin, hβ_2 adrenergic receptor, and turkey β_1 adrenergic receptor. In *grey* are highlighted the transmembrane regions, in red the highly conserved residues and in yellow cysteines that form disulfide linkages that involve EL2. For A_1, A_{2B}, A_3ARs only the cysteine residues that form the conserved disulfide bridge between TM3 and EL2 are highlighted in yellow, because information about other disulfide bonds is not available

The cysteine in EL2 that forms the disulfide bridge with TM3 is conserved, as well as the cysteine residue within TM3, and the linkage between these residues is also conserved. No mutagenesis data are available for the other cysteines.

On the A_{2A}AR there are another four cysteines that are connected by two disulfide bridges: Cys71-Cys159 and Cys74-Cys146. These residues correspond to Cys72, Thr162, Phe75 and Cys154 respectively on the A_{2B}AR, if we consider the alignment that allows the higher percentage of identity. In this case no other disulfide bonds are formed, and only one cysteine of EL2 is involved in a disulfide linkage, that is the one with TM3 that is conserved among GPCRs. In addition, there are two more cysteine residues in EL2 (Cys166 and Cys167); depending on the alignment, one of these residues can be aligned with Cys159 of A_{2A}AR and form a second disulfide bond that connects EL2 with Cys72 of the A_{2B}AR. It remains to be clarified how many disulfide bonds are actually present in the structure of the hA_{2B}AR. Nevertheless, the presence of three disulfide links on EL2 is a peculiarity of the hA_{2A}AR. This is an important point that has to be considered when the A_{2A}AR serves as a template for the homology modeling of other ARs to be used in drug design. The conformation of the A_{2A}AR binding pocket is influenced by EL2, which is strictly dependent on the presence of three disulfide linkages.

8.3 A_3AR Models

Homology models of the A_3AR have been helpful in providing structural hypotheses for the design of new ligands. Different A_3AR models have been published describing the hypothetical interactions with known A_3AR ligands having different chemical scaffolds, and almost all of these models were constructed using bovine rhodopsin as a template. As we have discussed before, the new structures of GPCRs solved in the past 2 years provide a new starting point for homology modeling. In particular, the recent publication of the A_{2A}AR provides important structural information for the AR family. Next to the structural information provided by the crystallographic data, mutagenesis studies can help to identify the residues that are involved in ligand recognition. Site-directed mutagenesis of the A_3AR shows an important role for specific residues in TM3, TM6 and TM7 (Chen et al. 2001; Duong et al. 2005; Gao et al. 2002a, b, 2003; Jacobson et al. 2001).

The three different models of the hA_3AR can be constructed using as templates: a. the bovine rhodopsin (PDB ID 1F88); b. the $h\beta_2$-adrenergic receptor (PDB ID 2RH1); and c. the hA_{2A}AR (PDB ID 3EML). The main differences between the templates are found within EL2, IL3 and the extracellular end of TM1 (Fig. 8.5). The structure-based drug design approach is mainly affected by differences in EL2, because residues of this loop can directly interact with ligands in the binding pocket. The EL2 of both squid and bovine rhodopsin assumes a β-sheet secondary structure, either in the structure with bound retinal or in the ligand-free structure. In the $h\beta_2$AR there is an α-helix in EL2 that is structurally similar to the β_1AR of turkey, while the A_{2A}AR does not have a defined secondary structure in the EL2.

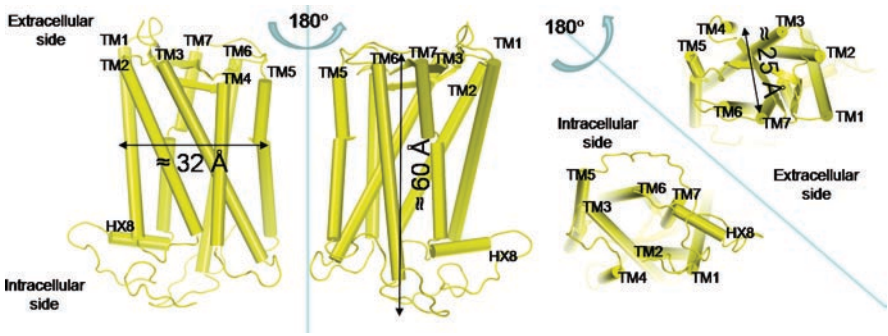

Fig. 8.2 Topology of the hA_3AR built using bovine rhodopsin as template

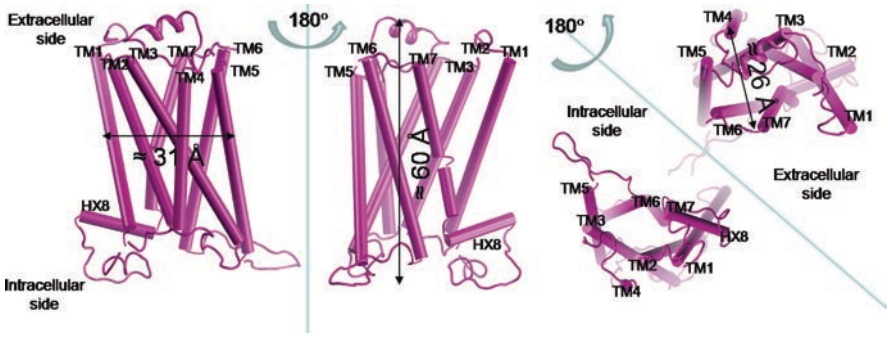

Fig. 8.3 Topology of the hA_3AR model built using the β2 receptor as template

The first model of the hA_3AR that we built was based on rhodopsin (Fig. 8.2). As for the high-resolution structure of rhodopsin, the hA_3AR model reveals a seven-helical bundle with a central cavity surrounded by helices 3, 5, 6 and 7. Helix 4 is not part of the cavity wall and makes contacts only with helix 3. The access to the central cavity is not allowed because the EL2 closes the binding pocket and determines a volume of the cavity of 660 $Å^3$. EL2 is characterized by a β-sheet secondary structure, and it is connected to TM3 with the conserved disulfide linkage between Cys83 and Cys166. This model has been widely used to identify putative ligand-receptor interactions and to understand and quantify the structure activity relationship (SAR) of known hA_3AR antagonists through a high-throughput docking strategy (Moro et al. 2005; Martinelli and Tuccinardi 2008).

Two other models of the hA_3AR were built using as a template the $h\beta_2$-adrenergic receptor and the turkey β_1-adrenergic receptor. The RMSD of the entire structures superimposed is approximately 4 Å, and it is 2.8 Å without considering the N-terminus (from residue 1 to 8), C-terminus (from residue 302 to 318), and IL3 (from residue 208 to 224), which are the most variable regions. The RMSD is only 1.8 Å considering only the helical backbone. These models do not present relevant differences at the active-site level, and therefore we are only considering the one built using the β_2-adrenergic receptor as the template (Fig. 8.3).

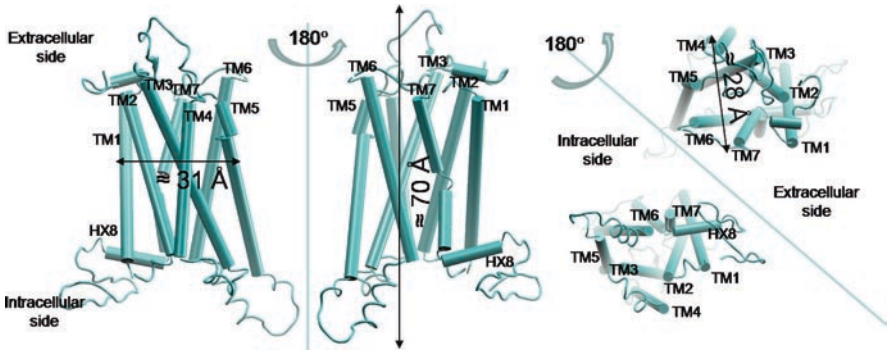

Fig. 8.4 Topology of the hA_3AR built using the A_{2A} receptor as template

Even though one of the two disulfide bridges in the EL2 is absent, the conformation of the EL2 of the hA_3AR model is similar to the EL2 of the adrenergic receptor template: an α-helical secondary structure enables the accessibility to the ligand-binding site. In the template, this conformation may be stabilized by an intra-loop disulfide bond, which is missing in the model of the hA_3AR. The putative location of ligands in the two templates is very similar. In preliminary docking studies, also the location of the hA_3AR antagonist is similar, even if there are structural differences in the ligand binding sites between the models obtained from rhodopsin and the adrenergic receptor. The largest difference within the TM region between the two models occurs in helix 1, in which the adrenergic receptor-based model lacks the proline-kink found in the rhodopsin-based model.

The recently published structure of the $hA_{2A}AR$ provides a new template for GPCR modeling and in particular for ARs. A new model of the hA_3AR was built using this crystal structure as the template (Fig. 8.4). The helical arrangement is similar among the models. However, the helices are shifted, and the differences among their relative positions result in an RMSD around 2.50 Å. As observed for the model built using adrenergic receptors as templates, the main difference in the helical bundle is TM1 and in particular the N-terminal end of the helix. A detailed comparison of the superimposed models is in Table 8.2, in which values of RSMD for each TM helix are reported. As was seen for the other templates, the main difference among the three models of the hA_3AR is in the loop region. The ligand binding pocket of the crystal structure of $A_{2A}AR$ is shifted closer to TM6 and TM7, and the position of the $A_{2A}AR$ antagonist ZM241385 is closer to these helices. Important interactions are also established with EL2. The position of ZM241385 is significantly different from the one of retinal or carazolol. Even though GPCRs share a common topology, ligands may bind in a different fashion and interact with different positions of the receptor. The model built starting from the $A_{2A}AR$ template is different from the previous models of A_3AR: the binding pocket is closer to TM6 and TM7 and open to the extracellular side. The volume of the binding sites of A_3AR models built starting from $h\beta_2$-AR and $hA_{2A}AR$ is difficult to be measured because these models present a binding site that is open to the extracellular side.

Table 8.2 Root mean square deviation (RMSD) of the backbone of the aligned models of the hA_3AR. The main difference among the models is due to the loops, which represent the most variable region of the templates and consequently of the models. Particular attention has to be given to EL2 because it is part of the binding pocket and it can directly interact with ligands

	All TMs	all loops	TM1	TM2	TM3	TM4	TM5	TM6	TM7	HX8	EL2
RMSD in Å with respect to the hA_3AR model from bovine rhodopsin (backbone)											
A_3–β2	2.29	10.86	2.82	2.12	1.98	2.01	2.07	2.19	1.85	3.73	11.44
A_3–A2A	2.43	10.06	2.55	2.40	2.78	2.45	2.85	2.02	2.04	1.64	14.30
RMSD in Å with respect to the hA_3AR model from $h\beta_2$-adrenergic receptor (backbone)											
A_3–rho	2.29	10.86	2.82	2.12	1.98	2.01	2.07	2.19	1.85	3.73	11.44
A_3–A2A	2.57	7.46	3.84	1.89	2.02	1.73	2.09	2.71	2.23	3.66	6.18
RMSD in Å with respect to the hA_3AR model from $hA_{2A}AR$ (backbone)											
A_3–rho	2.43	10.06	2.55	2.40	2.78	2.45	2.85	2.02	2.04	1.64	14.30
A_3–β2	2.57	7.46	3.84	1.89	2.02	1.73	2.09	2.71	2.23	3.66	6.18

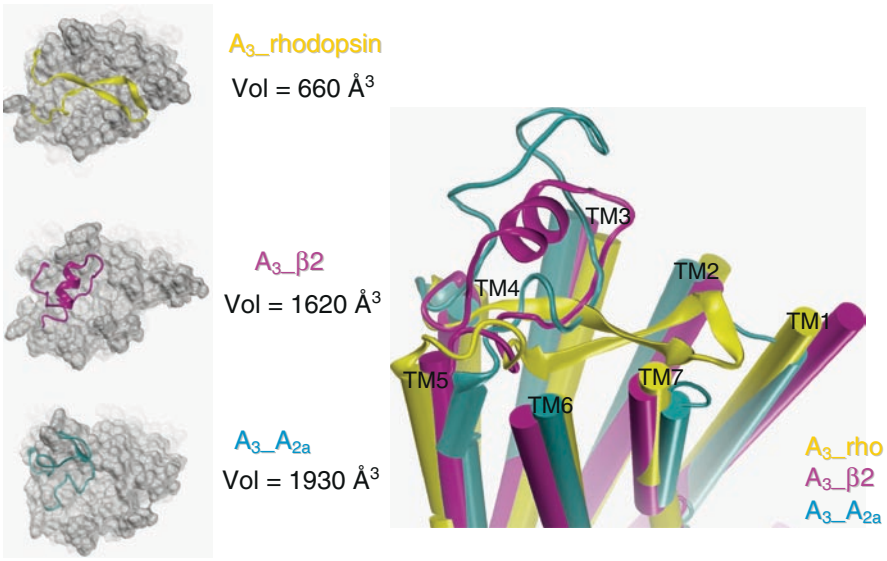

Fig. 8.5 A comparison of A_3AR models based on bovine rhodopsin, the human β_2-adrenergic receptor, and the A_{2A}AR as templates. On the *left*: Extracellular side view of hA_3AR models based on different templates and estimated volume of the binding pockets. On the *right*: Representation of EL2 of each hA_3AR model (in *yellow* hA_3AR built from rhodopsin, in *magenta* hA_3AR built from β_2-AR and in *cyan* hA_3AR built from hA_{2A}AR)

The volumes were estimated as 1,620 and 1,930 Å^3, respectively, but they cannot be compared with the volume of the binding site of the rhodopsin-based model, which is closed and has a volume of 660 Å^3 (Fig. 8.5).

Even if the percentage of identity of the hA_3AR is higher with respect to the A_{2A}AR than with the previously reported structures, the conformation of the EL2 and consequently of the binding pocket of the hA_3AR might be different from the A_{2A}AR. The peculiarity of the A_{2A}AR is the presence of three disulfide bridges on EL2, which are not conserved among ARs. Also, the particular conformation of EL2 and the binding pocket can be unique to this subtype, and use of the A_{2A}AR as a template for modeling other AR subtypes is still imprecise. Also, mutagenesis data support the hypothesis of different roles of TM helices in different AR subtypes.

One more disulfide bridge is present in the crystal structure of the hA_{2A}AR between Cys259 and Cys262 on EL3. A_3AR and A_{2B}AR do not present cysteine residues in the same positions, and the link cannot be formed in these two receptor subtypes. According to the alignment of the sequences, A_1AR presents two cysteines (Cys260 and Cys263) in the corresponding positions of the cysteines of A_{2A}AR: the formation of the disulfide bond is possible, but mutagenesis analysis shown that mutation of cysteine residues to alanine or serine does not change the affinity for agonists or antagonists.

8.4 Reengineered A_3ARs: Neoceptors

The clinical use of selective AR agonists has been limited by side effects due to less than complete specificity of a given ligand for its intended subtype. For example, high doses of the A_3AR agonist IB-MECA, a compound intended for treatment of rheumatoid arthritis, have produced cardiovascular side effects in some patients. Furthermore, due the widespread distribution of the ARs throughout the body, even a subtype specific agonist would be expected to have side effects. We have introduced an alternative approach to the administration of synthetic agonists that acting alone at the native receptors, which would avoid the inherently nonselectivity resulting from the wide distribution of the native ARs. This approach, which has been termed neoceptors, combines synthetic medicinal chemistry and genetic modification of the target proteins. By this approach, the putative ligand binding site of a given GPCR, as characterized using molecular modeling and other structural information, is reengineered for activation by a synthetic tailored agonist (a small molecule, neoligand) that is designed to have a structural complementarity to a mutated receptor. Thus, molecular modeling is a critical element in the design of reengineered receptors, that is introduction of a strategic mutation in the receptor to produce a neoceptor. This neoceptor is designed for selective activation by a novel synthetic neoligand at concentrations that do not activate the native receptor. If complete orthogonality is achieved, the neoligand is inactive at the native receptor at any concentration, and the neoceptor would be dormant until activated by the exogenously administered small molecule neoligand. It is possible to achieve this complementarity through the mutation of a single amino acid residue that is predicted to be in close proximity to a functional group of the agonist ligand that has been specfically modified. The modifications are done in a complementary fashion so that the two groups exhibit a novel mode of interaction, for example, reversing the polarity in a salt bridge, introducing unique hydrogen bonding sites, or creating a bump and hole (Jacobson et al. 2007). If a stabilizing interaction such as hydorgen bonding exists between the two complementary groups, an increase in affinity is expected at the mutant receptor based on this direct physical interaction.

With the neoceptors derived from the A_3AR, the intention is to make use of the cytoprotective properties of this receptor (for example in ischemic heart muscle tissue). To satisfy the requirements of this strategy, it is necessary that the neoligand not only bind to but also activate the neoceptor. Furthermore, the particular effector pathway that is associated with the desired beneficial effect, among the multiple pathways downstream of a given GPCR, must be activated. This strategy is intended for eventual use in gene therapy and may also be useful in mechanistic elucidation, using neoceptor-neoligand pairs that are pharmacologically orthogonal with respect to the native species.

Neoceptors have so far been applied successfully to the A_3AR and to other ARs (Gao et al. 2006, Jacobson and Gao 2006; Jacobson et al. 2005). Structural modification of the ribose moiety, which binds in a hydrophilic region of the putative ligand binding site of the receptor, has been region of adenosine that is most suited for modification for neoceptor recognition. It was predicted previously in various

modeling studies that the 2′ and 3′ hydroxyl groups of adenosine are in proximity to TM7, and specifically to the imidazole side chain of H272. This His residue is conserved among the four ARs and is proposed to be important in ligand recognition. Nucleoside analogues that were modified at the position of the 3′-OH were found to interact selectively with a H272E mutant hA_3AR (neoceptor). For example, intially we replaced the 3′-OH group of adenosine with a simple amino group, which produced a gain in affinity of ~sixfold at the H272E neoceptor.

Optimization of this enhancement effect required different groups to be placed at the 3′ position. In addition, structural changes were made at other points on the nucleoside structure. For example, adenosine N^6-benzyl derivatives were optimized for recognition in the relatively hydrophobic portion of the putative binding site of the hA_3AR, and this optimization generalized to the H272E neoceptor. Replacement of the 3′-OH group of substituted N^6-benzyladenosine analogues with a 3′-(aminomethylene) group resulted in a ~20-fold gain in affinity at the same neoceptor. Replacement of the 3′-OH group with a urea group in the case of N^6-benzyladenosine analogues produced an even larger gain in affinity of ~200-fold, leading to a Ki value of 220 nM in the case of MRS3481 (N^6-(3-iodobenzyl)-2′-deoxy-2′-ureidoadenosine). The urea group is capable of donating and accepting multiple hydrogen bonds, and a bidentate mode of recognition by carboxylate group of the Glu272 residue was proposed, based on a molecular model. The enhanced affinity of the 3′-ureido-N^6-benzyl adenosine derivatives such as MRS3481 was specific for the H272E mutation of TM7. Similar glutamate substitution of two other residues, T94E in TM3 or Q167E in EL2, failed to enhance the affinity of the urea-modified ligands (K_i values were >10 μM). This provided evidence of spatial proximity of the 3′-OH group of adenosine and its analogues with H272, as predicted by the molecular modeling.

8.5 Conclusions

The investigation of A_3AR and its ligands is rapidly growing with an increasing impact in the drug discovery process. There is now extensive evidence for the involvement of the A_3AR in the aetiology of many diseases, and therefore discovering novel agonists and antagonists for this receptor subtype is strongly warranted. The development of ligands for the A_3AR has been directed mainly by traditional medicinal chemistry, but the influence of structure-based approaches is increasing. Rhodopsin-based homology modeling had been used for many years to obtain three-dimensional models of the A_3AR, and different A_3AR models have been published describing the hypothetical interactions with known A_3AR ligands having different chemical scaffolds. The recently published structure of the human $A_{2A}AR$ provides a new template for GPCR modeling. However, even use of the $A_{2A}AR$ as a template for modeling other AR subtypes is still imprecise. Models based on bovine rhodopsin, the human β_2-adrenergic receptor, and the $A_{2A}AR$ are compared. The sequence of the human A_3AR contains only one cysteine residue (Cys166) in the second extracellular look (EL2), which putatively forms a conserved disulfide bridge with

the respective cysteine residues of TM3 (Cys83). Site-directed mutagenesis of the A_3AR shows an important role in ligand recognition for specific residues in TM3, TM6 and TM7. The approach of neoceptors is a means of reengineering a given GPCR, such as the A_3AR, to recognize a chemically tailored agonist ligand, and to no longer recognize the native agonist. It can serve to validate a molecular model, by establishing proximity of functional groups in the pair of neoceptor and its complementary tailored agonist ligand, assuming that a neoceptor-neoligand pair is pharmacologically orthogonal with respect to the native species. It may also be useful in mechanistic elucidation and is projected for future use in gene therapy.

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Part V
Effects on Tissues and Organs and Therapeutic Applications

Chapter 9
Adenosine A_3 Receptor Signaling in the Central Nervous System

Felicita Pedata, Anna Maria Pugliese, Ana M. Sebastião, and Joaquim A. Ribeiro

Abbreviations

AK	Adenosine-kinase
AD	Anoxic depolarization
aCSF	Artificial cerebrospinal fluid
APNEA	N(6)-2-(4 Aminophenyl)ethyladenosine
AR	Adenosine Receptor
AR132	N^6-methyl-2-Phenylethynyladenosine
CADO	2-Chloroadenosine
CNS	Central nervous system
CCL-2	Chemokine (C-C motif) ligand 2
cAMP	Cyclic AMP
CHA	N6-cyclohexyladenosine
Cl-IB-MECA	1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-p-yl]-1-deoxy-N-methyl-beta-D-ribofuranuronamide
EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride
ERK1/2	Extracellular signal-regulated kinases
NECA	5-N-Ethylcarboxyamidoadenosine
D-PIA	D(-)N(6)-(2-Phenylisopropyl)adenosine
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine

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$GABA_A$	Gamma-aminobutyric acid A
GFAP	Glial fibrillary acidic protein
KO	Knockout
5-HT	5-Hydroxytryptamine
IB-MECA	1-Deoxy-1-[6-[[(3-iodophenyl)-methyl]amino]-9H-purin-9-yl]-N-methyl-beta-D-ribofuranuronamide
LJ1251	(2*R*,3*R*,4*S*)-2-(2-chloro-6-(3-iodobenzylamino)-9H-purin-9-yl) tetrahydrothiophene-3,4-diol
L-PIA	L(-)N(6)-(2-Phenylisopropyl)adenosine
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
Map-2	Microtubule-associated protein 2
MCAo	Middle cerebral artery occlusion
MAPK	Mitogen activated protein kinase
MRS1191	3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(+/−)-dihydropyridine-3,5-dicarboxylate
MRS1220	9-Chloro-2-(2-furanyl)-5-((phenylacetyl)amino)-[1,2,4]triazolo[1,5-c]quinazoline
MRS1340	1,4-Dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyr idinedicarboxylic acid 3-ethyl-5-[(3-nitrophenyl)methyl] ester
MRS1523	5-Propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate
NMDA	N-Methyl-D-aspartate
NBTI	S-(4-Nitrobenzyl)-6-theoinosine
OGD	Oxygen–glucose deprivation
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PLC	Phospholipase C
RT-PCR	Reverse transcription–polymerase chain reaction
SERT	Serotonin-selective reuptake transporter
TNF alpha	Tumour necrosis factor alpha
VT72	N^6-Methoxy-2-phenylethynyl
VT158	N^6-Methoxy-2-phenylethynyl
VT160	N^6-Methoxy-2-(2-pyridinyl)-ethynyl
VT163	N^6-Methoxy-2-*p*-acetylphenylethynyl

9.1 Introduction

In 1972 Ginsborg and Hirst (1972) described for the first time that the nucleoside adenosine inhibited acetylcholine release at the rat-diaphragm neuromuscular junction and that this effect was abolished by theophylline. This observation was soon supported by Ribeiro and Walker (1975) using the frog neuromuscular junction preparation.

Authors discussed these results in relation to the capability of adenosine to increase cyclic AMP (cAMP), an effect that was abolished by theophylline as previously demonstrated by Sattin and Rall (1970). In 1979 van Calker et al. (1979) proposed that adenosine regulates the accumulation of cAMP in cultured brain cells, via two different types of adenosine receptors and in 1980, Londos et al. (1980) proposed subclasses of membrane adenosine receptors: the A_1/Ri (that inhibited adenylate cyclase) and the A_2/Ra (that activated adenylate cyclase). In 1984 Ribeiro and Sebastião (1984) in an attempt to characterize the type of adenosine receptors involved in the inhibitory action of adenosine at the frog neuromuscular junction, suggested that the adenosine receptors at the frog neuromuscular junction should not be classified as A_1/A_2 because the potency profile of adenosine agonists did not fit the pharmacological profile proposed for either A_1 or A_2 receptors. As a consequence, Ribeiro and his Ph.D. student, Ana M. Sebastião, were requested by the editors of Progress in Neurobiology to write a review on adenosine receptors. In reviewing the available pharmacological information, three different adenosine receptor entities emerged based on the affinity of different adenosine agonists: A_1 with a pharmacological profile with L-PIA, CHA>CADO>D-PIA, NECA (high stereoselectivity for the PIA isomers) and negatively coupled to adenylate cyclase; the A_2 with NECA>CADO>L-PIA, CHA, D-PIA with low stereoselectivity for the PIA isomers (L-PIA~D-PIA) and positively coupled to adenylate cyclase. A third entity was then proposed, an A_3 adenosine receptor (A_3AR) with an agonist profile with L-PIA, CHA, NECA>CADO, and D-PIA usually less potent than CADO, possibly linked to calcium (Ribeiro and Sebastião 1986).

After cloning, it was clarified that rat A_3AR is homologous with the adenosine A_1 and A_{2A} receptors and belongs to the G-protein-coupled receptor family (Meyerhof et al. 1991a, b). After cellular expression of cloned A_3AR and pharmacological characterization, rat A_3AR emerged as having a very low affinity for xanthine-based adenosine receptor antagonists such as theophylline. Various specie homologues of this receptor have been cloned, including the human A_3AR (Salvatore et al. 1993). It emerged that A_3AR cloned from different species show different pharmacological properties. Cloning and expression of the human A_3AR stably expressed in Chinese hamster ovary cells pointed out that, contrary to rat A_3AR, human A_3AR is xanthine sensitive.

9.2 Distribution of A_3AR in the Central Nervous System (CNS)

The first binding studies on solubilized membranes from rat brain demonstrated the presence of a low affinity adenosine receptor with characteristics of the A_3 subtype (Oliveira et al. 1991). However, in situ hybridization studies in the rat indicated the presence of A_3AR mRNA only in the testis (Meyerhof et al. 1991a; Zhou et al. 1992; Rivkees 1994) and not in the CNS (Rivkees et al. 2000). Similarly, no expression of A_3AR in the brain of mice or in the hippocampi of humans was detected (Rivkees et al. 2000). However, by reverse transcription–polymerase chain reaction (RT-PCR)

A_3AR expression was found distributed in the rat heart, lung and widespread in the rat and mouse brain (Zhou et al. 1992; Dixon et al. 1996; von Arnim et al. 2000). Low levels were detected by A_3AR binding in various regions of the mouse brain (Jacobson et al. 1993), more than five times below that of the A_1 receptor (Cunha et al. 1995) or of the A_{2A} receptor (Cunha et al. 1996a).

There are significant brain regional differences in the levels of A_3AR mRNA. In the mice, there is evidence for the expression of the A_3AR in the hippocampus, thalamus and hypothalamus (Yaar et al. 2002). It is generally accepted that A_3AR has species-specific tissue distribution. In humans and sheep, A_3AR is significantly expressed in many peripheral tissues with lower levels in the CNS and testis (Linden et al. 1993; Salvatore et al. 1993).

As to A_3AR localization in the different cell type of the CNS, the presence of A_3AR in neurons, primarily at presynaptic sites, was demonstrated by PCR of laser dissected hippocampal neurons and by western blotting in rat hippocampal nerve terminal membranes (Lopes et al. 2003). Moreover, A_3AR mRNA is expressed in microglia (Fiebich et al. 1996) and has been identified by Northern blot analysis in mouse astrocytes (Zhao et al. 1999).

9.3 The Roles of A_3AR in the CNS

The endogenous neuromodulator, adenosine, controls and integrates a wide range of brain functions; its extracellular levels vary according to behavioral state and pathophysiological condition. Dysfunction of the adenosine system is involved in pathologies ranging from epilepsy to neurodegenerative disorders and psychiatric conditions. Less is known about the contribution of the low-affinity A_3AR to the regulation of brain function and neuropathological conditions if compared to high-affinity A_1 and A_{2A} adenosine receptors. Its role in several pathophysiological conditions is often enigmatic and controversial.

9.3.1 Role of A_3AR in Memory and Cognition

Phenomena of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) are the likely cellular substrates for learning and memory. It is possible to induce either LTP or LTD according to the magnitude of the transient calcium levels attained at restricted synaptic spine domains. Smaller calcium increases predominantly activate protein phosphatases, leading to LTD, whereas higher calcium levels activate protein kinases, causing LTP (Dudek and Bear 1993).

Adenosine affects synaptic plasticity phenomena acting on both A_1 and A_{2A} receptors (de Mendonca and Ribeiro 2000; Rebola et al. 2008). The estimated affinity of rat A_3AR for the endogenous ligand, adenosine (Ki = 1 μM), is considerably lower compared to that of A_1 receptors (Ki = 10 nM) or A_{2A} receptors (Ki = 30 nM)

(Jacobson et al. 1995). Since the extracellular concentration of endogenous adenosine does not exceed 300 nM (Latini and Pedata 2001) it is difficult to detect a physiological role of A_3AR in the CNS. However, conditions of stimulation that elicit synaptic plasticity can also transiently raise the extracellular adenosine concentration three to tenfold over basal levels (Cunha et al. 1996b). A role for A_3AR on LTP and LTD has also been reported in the hippocampus (Costenla et al. 2001). Activation of A_3AR essentially attenuates LTD (Costenla et al. 2001; Huang et al. 2007) and allows induction of LTP. The facilitating effects of the A_3AR agonist, (1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-p-yl]-1-deoxy-N-methyl-beta-D-ribofuranuronamide (Cl-IB-MECA) on LTP were observed with weak subliminal θ-burst induction conditions but not with high frequency stimulation, which would elicit marked postsynaptic depolarization and Ca^{2+} increase, thus overcoming the facilitory effect of A_3AR activation. This effect is consistent with a modification in the threshold for the induction of long-term synaptic changes, and might be due to coupling of A_3AR to the phospholipase C transducing pathway (Abbracchio et al. 1995), thus promoting a shift to increased Ca^{2+} levels and protein kinase activation. The Cl-IB-MECA effect is prevented by the selective A_3AR antagonist, MRS1191, indicating a genuine A_3AR mediated response. However, the A_3AR antagonist, MRS1191, does not by itself modify θ-burst-induced LTP, suggesting that tonically released adenosine is not able to activate A_3AR in order to modulate LTP.

Behavioral studies have confirmed the involvement of A_3AR in spatial learning and memory. In female Swiss mice, 1-deoxy-1-[6-[[(3-iodophenyl)-methyl]amino]-9H-purin-9-yl]-N-methyl-beta-D-ribofuranuronamide (IB-MECA), administered i.p. 20 min before tests, diminished scopolamine- and MK-801-induced impairment of spontaneous alternation in Y-maze and learning abilities in a passive avoidance task indicating that A_3AR stimulation may ameliorate spatial memory and long term memory impairments in terms of cholinergic and glutamatergic deficits (Rubaj et al. 2003). It must be mentioned, however, that in the same animal species, Borowicz et al. (1997), using the passive avoidance task, demonstrated that a non selective A_3AR agonist, N(6)-2-(4 aminophenyl)ethyladenosine (APNEA), administered i.p. 30 min before behavioral tests, impaired long-term memory.

When looking at A_3AR-mediated behavioral effects, it should be remembered that A_3AR agonists have depressant effects on locomotor activity (Jacobson et al. 1993).

9.3.2 Role of A_3AR in Locomotion

By studying locomotor activity in an open field, Jacobson et al. (1993) demonstrated that intraperitoneal administration of 3-IB-MECA in mice induces a depression of motor activity. These results are consistent with the most recent experiments performed in A_3AR-deleted mice. Significant increases in some aspects of motor function were observed in A_3AR-deleted mice by using three different tests: activity in the open field; number of arm entries in the elevated-plus maze; and number

of transitions in the light/dark box (Fedorova et al. 2003). The change in motor activity appears selective without evidence of ataxia. The increase in motor activity was attributed to disinhibition of cortical neurons because selective A_3AR stimulation inhibits excitatory neurotransmission in rat cortical neurons (Brand et al. 2001). Most recently it was confirmed that both adolescent (21-day old) and adult A_3AR knockout (KO) mice showed an increase of motor activity in the open field (Bjorklund et al. 2008a). Moreover, a reduced response to the motor-stimulating effect of caffeine or amphetamine was found in A_3AR KO mice. These data are surprising in view of the poor affinity of A_3AR for caffeine (Bjorklund et al. 2008a).

Work reviewed above indicates that even though A_3AR are expressed at low level in the central nervous system, they play a tonic role in modulating motor activity.

9.3.3 Role of A_3AR Receptors in Convulsions

Adenosine was identified as an endogenous anticonvulsant in the brain more than 20 years ago (Dunwiddie 1980; Lee et al. 1984; Dragunow et al. 1985; Dragunow 1991) and it was suggested that dysfunction of the adenosine-based neuromodulatory system might contribute to epileptogenesis (Boison 2007, 2008). Many experimental convulsive procedures led to a considerable rise in extracellular levels of adenosine (for a review see: Boison 2008). Clear-cut evidence that this really occurs in epileptic patients was provided by During and Spencer (1992). Their studies, with microdialysis probes implanted in the hippocampi of epileptic patients with intractable complex partial epilepsy, revealed that extracellular levels of adenosine in the dialysate were elevated by six- to 31-fold during seizures.

Recently, the therapeutic potential of stem cells engineered to release adenosine as a local source to augment endogenous adenosinergic functions was assayed in two cell transplantation experiments (Li et al. 2007, 2008; Boison 2008). Most of the studies about the role of adenosine as an anticonvulsant emphasize the preeminent involvement of A_1 adenosine receptors. However, several studies using different experimental models of epilepsy have investigated the role of adenosine A_{2A} and A_3 receptors in this condition.

A first report conducted in an in vivo seizure model in mice indicates that stimulation of A_3AR protects from seizures (von Lubitz et al. 1995). Acute systemic administration of IB-MECA protects against chemically-induced (NMDA injection) but not electrically-induced seizures while a protective effect of chronically administered IB-MECA is evident in both chemically- and electrically-evoked seizures (von Lubitz et al. 1995). The protective effect of acute administration of IB-MECA is attributed to both arteriolar constriction and severe hypotension (von Lubitz et al. 1994), which can reduce the final intracerebral concentration of the chemoconvulsant NMDA (von Lubitz et al. 1995). In a study conducted on seizure-sensitive DBA/2 mice, an animal model of generalized

reflex epilepsy, the intraperitoneal administration of A_3AR agonist IB-MECA is without effect (De Sarro et al. 1999). More recently, Vianna et al. (2005) demonstrated in adult rats in status epilepticus (SE) induced by pilocarpine, that pretreatment with the A_3AR antagonist MRS1220 does not alter the incidence of SE but reduces the latency to develop it.

Although the above reviewed evidence would support the concept that A_3AR exerts a protective role against seizures, in some cases data indicates that A_3AR stimulation by endogenous adenosine may aggravate epileptic activity, as determined in CA1 area of rat immature hippocampal slices in which seizure activity was induced by Mg^{2+} deprivation together with brief high frequency stimulation of Shaffer collaterals (Etherington and Frenguelli 2004). Such an A_3AR-mediated excitatory effect is in agreement with the notion that activation of A_3AR in the adult hippocampus exerts excitatory effects, that is increases high-threshold calcium currents (Fleming and Mogul 1997), desensitizes the inhibitory A_1 receptors (Dunwiddie et al. 1997) and inhibits presynaptic inhibitory metabotropic glutamate receptors (Macek et al. 1998). In addition, 2-Cl-IB-MECA increases the amplitude of electrically evoked and the frequency of spontaneous epileptiform field potentials recorded during $GABA_A$ receptor blockade in CA3 area of rat immature hippocampal slices (Laudadio and Psarropoulou 2004). An enhanced amplitude of evoked responses associated with the increase in frequency of spontaneous discharges may facilitate seizure-induced neuronal damage. The frequency of spontaneous discharges recorded during $GABA_A$ and A_1 or A_{2A} receptor blockade is not affected by the A_3AR antagonist, MRS1220 (Laudadio and Psarropoulou 2004). This indicates that adenosine, tonically released from immature slices, is unlikely to increase the rate of spontaneous discharges. However, the high affinity uptake blocker NBTI (Deckert et al. 1988), which increases extracellular adenosine, induced spontaneous discharges in a subset of rat immature slices (Laudadio and Psarropoulou 2004). Two lines of evidence suggest that this effect is mediated by A_3AR. First, 2-Cl-IB-MECA has no additional excitatory effect following an NBTI-induced excitation, and secondly, the NBTI-induced increase in the frequency of spontaneous discharges (in the presence of the A_1 antagonist DPCPX) was reversed by the A_3AR antagonist MRS1220. The NBTI-induced excitation in immature slices is a novel finding which may be peculiar to the developing hippocampus. In fact in the adult hippocampus NBTI has an inhibitory effect on evoked or spontaneous discharges; this is in line with the consistent A_1-mediated inhibitory effects of adenosine (Sanderson and Scholfield 1986).

The variable effects of NBTI in the immature and mature hippocampus might be ascribed to changes in the levels of endogenous adenosine (Park et al. 1987) and/or in the proportion of A_1 and A_3AR.

In conclusion, the excitatory effects of A_3AR activation on synchronous epileptiform discharges in vitro suggest that an increase of endogenous adenosine in conditions of stress, that is seizures or hypoxia, may enhance synaptic activity in the immature brain. In addition, they raise the possibility that the A_3AR subtype may play a role in the establishment of activity-dependent plastic changes.

The blockade of A_3AR with the selective antagonist, MRS1334, improves the stability of $GABA_A$ergic neurotransmission, as assessed in a patch clamp study on $GABA_A$ receptor isolated from neurosurgically resected epileptic human nervous tissues and microtransplanted into Xenopus oocytes, and on human epileptic slices obtained from neurosurgical resection (Roseti et al. 2008).

Therefore, antagonism of A_3AR may increase the inhibitory efficacy of $GABA_A$ receptor in some forms of human epilepsy, pointing towards new therapeutic targets to fight epilepsy.

9.3.4 Role of A_3AR in Nociception

Adenosine exerts complex influences on pain transmission by different mechanisms in the brain and spinal cord, as demonstrated in a broad spectrum of animal pain models (for a review see: Sawynok 1998). Several therapeutic approaches to pain and inflammation based on mimicking or modulating the effects of endogenous adenosine are currently under preclinical and clinical investigation. These include the use of adenosine itself, the use of direct-acting adenosine receptor agonists and the use of agents designed to modulate the levels and therefore the actions of adenosine in the extracellular space (adenosine-kinase (AK) inhibitors) (see: Gao and Jacobson 2007). Much evidence indicates that adenosine receptor activation in the spinal cord produces antinociception due to stimulation of A_1 receptors. This effect is attributed to presynaptic inhibition of excitatory neurotransmitter release with subsequent reduction of substance P concentration, as detected in cerebrospinal fluid (Sjolund et al. 1997) and therefore due to postsynaptic inhibition of the glutamate effects (DeLander and Wahl 1988).

A_3AR ko have decreased nociception, as assessed by the hot-plate test (Fedorova et al. 2003), probably due to a decrease in the supraspinal processing and "recognition" of painful stimuli. This is consistent with the localization of A_3AR in thalamic nuclei (Yaar et al. 2002) where they may play a role in processing nociceptive information.

Following carrageenan-induced inflammation in the hind paw, heat hyperalgesia, plasma extravasation and edema were significantly reduced in A_3AR-deleted mice compared to wild type mice (Wu et al. 2002) suggesting that A_3AR plays a role in generating the localized inflammatory response which is in agreement with previous evidence that adenosine A_3AR activation produces pain behaviors secondary to mast cell degranulation and release of histamine and 5-hydroxytryptamine (5-HT) that exert nociceptive actions at sensory nerve terminal (Sawynok et al. 1999). Subcutaneous administration of adenosine A_3AR agonists produces nociceptive behavior (Sawynok et al. 1997).

The above data support the pro-nociceptive role of A_3AR involved in both central nervous system effects and pro-inflammatory effects on peripheral tissues.

It must be mentioned, however, that intrathechal administration of IB-MECA does not exhibit an antinociceptive profile in acute nociception as assessed in the early phase pain response of the formalin test, but it does depress the late phase of

prolonged pain that measures hyperalgesia related to formalin-induced inflammation (Yoon et al. 2005, 2006). This suggests an involvement of spinal adenosine A_3AR in protection from nociception and is in agreement with the observation that A_3AR agonists control the in vitro release of pain-related neuropeptides from the rat spinal cord (Mauborgne et al. 2002).

9.3.5 *Role of A_3AR in Mood and Affects*

Miller and Hoffman (1994) demonstrated that activation of A_3AR results in increased 5-HT-uptake in rat basophilic leukemia cells. Likewise, but in rat central nervous system, the activation of adenosine A_3AR by the agonist APNEA during adenosine A_1 receptor blockade, decreases hippocampal extracellular 5-HT levels in freely moving rats (Okada et al. 1997). The inhibitory effect of the A_3AR agonist on extracellular 5-HT levels is abolished by inhibition of 5-HT reuptake activity with DU24565 and fluoxetine (Okada et al. 1999). It was later established using rat basophilic leukemia 2H3 cells, that activation of A_3AR, via both PKG and p38 MAPK, stimulates the activity of 5-HT transporter (SERT) (Zhu et al. 2004) that is the main responsible for inactivation of synaptic 5-HT and a main target of antidepressant drugs which inhibit SERT activity.

More recently, it has been reported that the A_3AR agonist IB-MECA rapidly (10 min) and selectively stimulates 5-HT transport in mouse midbrain, hippocampal, and cortical synaptosomes (Zhu et al. 2007). IB-MECA-induced stimulation of 5-HT uptake is blocked by the selective A_3AR antagonist MRS1191 and is absent in synaptosomes prepared from A_3AR-knockout mice (Zhu et al. 2007). In view of these results, a possible antidepressant effect of A_3AR antagonists could be envisaged. However such extrapolation is not supported by behavioral depression tests evaluated in A_3AR deleted mice. A_3AR ko mice show an increase in the amount of time spent immobile in two tests of behavioral depression, the forced-swim test and the tail-suspension test, respectively (Fedorova et al. 2003). This response is probably not attributable to a decrease in motor activity, mainly because of the increased locomotion expressed by the A_3AR deleted genotype (Fedorova et al. 2003).

Although there is evidence that A_3AR ko mice are more prone to depressive behavior, it is also reported that they have increased performance in the elevated-plus maze and light/dark box suggestive of reduced anxiety but this is most probably a consequence of the increase in exploratory activity due to increased motor activity (Fedorova et al. 2003).

9.3.6 *A_3AR and Cerebral Blood Flow Regulation*

One important issue not always sufficiently clarified is whether modification of blood pressure contributes to some effects of systemically administered A_3AR agonists. Von Lubitz et al. (1994) have shown that preischemic administration of IB-MECA results in a significant delay in the return of postischemic blood flow,

and this may affect neuronal survival (see Section 9.4.1). The hypotensive response observed following adenosine A_3AR activation in the anaesthetized rat may involve mediator release from mast cells (Fozard and Carruthers 1993; Fozard et al. 1996). Indeed A_3AR activation results in rapid mast cell degranulation in the anaesthetized rat. Moreover, a direct central regulation of arterial blood pressure is suggested by the study of Stella et al (1998).

9.4 Role of A_3AR in Neurodegeneration

There is major evidence regarding the role of A_3AR in neurodegenerative phenomena from in vivo and in vitro studies in hypoxia/ischemia models.

Although at relatively low levels in comparison to A_1 and A_{2A} receptors, A_3AR mRNA was detected in the rat and mouse brain by RT-PCR (Dixon et al. 1996) and radioligand binding (Jacobson et al. 1993). This receptor is widespread in the rat and mouse brain (see: Gessi et al. 2008). In comparison to A_1 and A_{2A} receptors, A_3AR has less affinity for adenosine (10–30 nM versus 1 μM). However, since extracellular adenosine concentrations increase dramatically during ischemia (Hagberg et al. 1987), evidence now indicates that all three adenosine receptors are potential targets for therapeutic treatment of stroke**.**

It is well accepted that by stimulation of A_1 receptors, adenosine exerts a protective role in ischemia by reducing Ca^{2+} influx by counteracting the presynaptic release of excitatory neurotransmitters (Corradetti et al. 1984). Moreover, by directly increasing the K^+ and Cl^- ion conductance, adenosine stabilizes neuronal membrane potentials, thus reducing neuronal excitability (Greene and Haas 1991). Furthermore, adenosine, through A_1 receptor activation, inhibits NMDA receptor-mediated currents (de Mendonca et al. 1995) and the NMDA receptor component of synaptic potentials during hypoxia (Sebastião et al. 2001). Reductions in synaptic transmission, in cellular metabolism and in energy consumption as well as moderate lowering of the body/brain temperature are protective in ischemia. Although data converge in demonstrating a neuroprotective effect of adenosine through A_1 receptors during ischemia, the use of selective A_1 agonists is hampered by unwanted peripheral effects, i.e. sedation, bradycardia, hypotension (Kafka and Corbett 1996). More recently, the role of A_{2A} receptors in ischemic neuroprotection has been studied. Most data report a beneficial effect evoked by A_{2A} antagonists that is attributed to reduced excitotoxicity and lower production of intracellular mediators involved in transcription mechanisms that may be relevant to neurodegeneration (Chen et al. 2007; Chen and Pedata 2008).

9.4.1 Role of A_3AR in Hypoxia/Ischemia

The studies currently in the literature concerning the role of A_3AR in the pathophysiology of cerebral ischemia are rather contradictory and have been matter of discussion

in several review papers (Jacobson 1998; Jacobson et al. 1999; Baraldi et al. 2000; von Lubitz 1999; von Lubitz et al. 1999, 2001).

An early in vivo study in the model of global forebrain ischemia in the gerbil showed that a selective agonist of A_3AR, IB-MECA, acutely administered 15 min prior to ischemia, impaired post-ischemic blood flow, increased mortality and exacerbated the loss of hippocampal neurons (von Lubitz et al. 1994). IB-MECA administration 20 min prior to transient middle cerebral ischemia also resulted in a significant increase in infarct size(von Lubitz et al. 2001).

In agreement with a possible noxious role of A_3AR in ischemia, it has been more recently shown in a model of in vitro ischemic preconditioning, that the selective A_3AR antagonist, 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate (MRS1523) applied before and during oxygen–glucose deprivation (OGD), facilitated the full recovery of CA1 hippocampal neurotransmission after a severe (7 min), irreversible OGD period (Pugliese et al. 2003). The harmful role of A_3AR during in vitro OGD is confirmed by the observation that the A_3AR selective antagonists MRS1523, the new antagonists, LJ1251 ((2R,3R,4S)-2-(2-chloro-6-(3-iodobenzylamino)-9H-purin-9-yl)tetrahydrothiophene-3,4-diol), the 2-arylpy razolo[3,4-c]quinoline and 4-modified-2-aryl-1,2,4-triazolo[4,3-a]quinoxalin-1-one derivatives and the 4-bismethanesulfonylamino-2-phenyl-1,2,4-triazolo[4,3-a] quinoxalin-1-one compound, consistently abolish or delay the occurrence of anoxic depolarization (AD) and significantly prevent the irreversible disruption of excitatory neurotransmission caused by a severe (7 min) ischemic episode (Pugliese et al. 2006, 2007; Colotta et al. 2007, 2008). The appearance of AD (Pugliese et al. 2006; Tanaka et al. 1997) is strictly correlated with the extent of brain damage during ischemia both in vivo and in vitro (Somjen 2001) and alterations in AD characteristics caused by A_3AR antagonists may be attributable to their actions on glutamate-mediated cellular responses (see: Pugliese et al. 2006, 2007). NMDA receptors are essential to AD initiation and propagation (Somjen 2001). The block of A_3AR, by removing A_3AR-mediated impairment of the feedback inhibition of glutamate release exerted by specific metabotropic glutamate receptor subtypes (Macek et al. 1998) may reduce the participation of glutamate in triggering the AD. Interestingly, Cl-IB-MECA facilitates epileptiform discharges in the CA3 area of immature rat hippocampal slices (Laudadio and Psarropoulou 2004), suggesting that following a rise of endogenous adenosine, as occurs during convulsions associated with hypoxia, A_3AR facilitates excitation, thus limiting the known protective inhibitory effect of adenosine in the brain. An A_3AR direct regulation of glutamate efflux appears unlikely on the basis of in vitro results showing that neither A_3AR activation with Cl-IB-MECA nor its blockade with MRS1191 modify neurotoxicity caused by kainate and cyclothiazide in cultured neurons (Rebola et al. 2005).

Contrary to the above information, Hentschel et al. (2003) demonstrated that under 5 min hypoxic conditions (95% N2–5% CO_2) in vitro, selective activation of A_3AR by a brief (5 min) application of IB-MECA, inhibits excitatory neurotransmission on cortical neurons. Such effect is blocked by the selective A_3AR antagonist MRS1220. These data indicate that A_3AR may sustain inhibition of synaptic activity during hypoxia and therefore mediate neuroprotection. Furthermore, IB-MECA

acutely administered 20 min after transient (30 min) focal cerebral ischemia decreases the infarct volume (von Lubitz et al. 2001). A possible protective role of A_3AR is supported by the observation that mice deleted for A_3AR showed more pronounced hippocampal pyramidal neuron damage following repeated episodes of moderate hypoxia (Fedorova et al. 2003) and an increase in cerebral infarction after transient ligation of the middle cerebral artery (Chen et al. 2006). In addition, intracerebroventricular or repeated intravenous administration (i.e., at 165 and 15 min before transient ligation of the middle cerebral artery) of Cl-IB-MECA decrease cerebral infarction assessed 2 days later. Cl-IB-MECA decreased the size of infarction in the wild-type controls, but not in the A_3AR knockout animals, confirming that Cl-IB-MECA-induced protection was mediated through the A_3AR (Chen et al. 2006). We must notice that the selective A_3AR antagonist, MRS1191, administered intracerebroventricularly 30 min before transient (60 min) middle cerebral artery occlusion (MCAo) did not modify the extent of infarction (Shen et al. 2005). This observation is in agreement with the poor role of A_3AR in normal physiological transmission. MRS1523 and LJ1251 do not in fact modify synaptic transmission in the CA1 area of the hippocampus under normoxic conditions (Pugliese et al. 2006, 2007).

These conflicting results on the excitatory or inhibitory role of A_3AR on synaptic activity under hypoxia/ischemia may be reconciled by recent data reported by Pugliese et al. (2007). The A_3AR antagonist, MRS1523, applied before a brief (2 min) OGD reduces the depression of CA1 hippocampal neurotransmission. This result indicates an inhibitory role of A_3 AR on synaptic transmission during brief OGD. Indeed a depression of synaptic activity such as that brought about by adenosine A_1 receptors during ischemia is considered neuroprotective. In fact, antagonists of A_1 receptors reduce inhibition of synaptic transmission, impair the recovery of synaptic potentials (Sebastião et al. 2001) and shorten the onset of AD induced by hypoxia in the CA1 region of hippocampal slices (Lee and Lowenkopf 1993). Results by Brand et al. (2001), Lopes et al (2003) and Pugliese et al (2007) indicate no interplay between A_3AR and A_1 receptors on the inhibition of excitatory transmission. The decrease in synaptic depression brought about by A_3AR antagonists after hypoxia or a brief OGD period suggests that A_3AR as A_1 receptor inhibit fEPSP amplitude during the first few minutes of OGD, therefore sharing a neuroprotective role with A_1 receptors.

Results suggest that in the first phase of ischemia A_3AR, by decreasing synaptic transmission, play a protective synergistic role with A_1 receptors. However, recovery of transmission after ischaemia may be influenced by several processes, some eventually unrelated to the level of synaptic transmission during hypoxia. Prolonged ischemic conditions could play a pivotal role in switching the effects of A_3AR stimulation from A_1-like inhibition to potentiation of an excitotoxic glutamate effect. The activation of phospholipase C (PLC) by A_3AR has been reported in striatal and hippocampal slices (Abbracchio et al. 1995). Rat cortical neurons exposed to hypoxia in vitro show an increase in activation of protein kinase C (PKC) after selective A_3AR stimulation (Nieber and Hentschel 2006). Similarly to what is described in the heart, PKC-dependent activation of K_{ATP} channels may enhance adenosine protection (Liang and Jacobson 1998), but if OGD is applied long enough to be considered severe, PKC activation induced by A_3AR could account for an increase

in intracellular calcium, which may participate in increasing tissue excitability and thus lead to irreversible synaptic failure.

Taking into account that during ischemic conditions, adenosine is released from hippocampal slices, reaching concentrations up to 30 μM after 5-min OGD (Latini et al. 1998; Pearson et al. 2006) and that A_3AR are stimulated by μM concentrations of adenosine (Fredholm et al. 2001) we may speculate that A_3AR mediated effects would become particularly deleterious during ischemia, when high levels of adenosine are reached extracellularly and detrimental effects of A_3AR activation may be due, at least in part, to increased excitoxicity.

Altogether these data suggest that the outcome of A_3AR stimulation on synaptic transmission during hypoxic/ischemic phenomena depends on the duration and severity of the ischemic episode. Although A_3AR may play a protective role in the first phase of ischemia, prolonged A_3AR stimulation by high adenosine concentrations could be pivotal in transforming the A_3AR-mediated effects from protective to injurious.

Interestingly, in the in vitro OGD model in hippocampal slices, it was found that a long application (before and during OGD) of 5′-N-methylcarboxamidoadenosine derivatives Cl-IB-MECA and of new selective A_3 agonists (Volpini et al. 2002, 2007): VT72 (N^6-methoxy-2-phenylethynyl), VT158 (N^6-methoxy-2-phenylethynyl), VT160 (N^6-methoxy-2-(2-pyridinyl)-ethynyl), VT163 (N^6-methoxy-2-*p*-acetylphenylethynyl) and AR132 (N^6-methyl-2-phenylethynyladenosine) have effects similar to those of antagonists upon the OGD-induced depression of synaptic transmission and on the appearance of AD after the severe (7 min) OGD period (Pugliese et al. 2007). These effects may be attributed to desensitization of A_3AR. In fact, both human and rat A_3AR are desensitized within a few minutes after agonist exposure (Palmer et al. 1995; Trincavelli et al. 2002). A stimulation as massive as that reached in the presence of endogenous adenosine plus exogenous A_3AR agonists might induce substantial A_3AR plastic adjustments such as desensitization. These in vitro results probably concord with the observation in the model of global forebrain ischemia in the gerbil (von Lubitz et al. 1994), that chronic administration (10-day pre-ischemic) of IB-MECA improves post-ischemic cerebral flow circulation, survival and neuronal preservation (von Lubitz et al. 1994, 1999) and that repeated intravenous administration of Cl-IB MECA before MCAo ligation increases locomotor activity and decreases cerebral infarction (Chen et al. 2006). Chronic preischemic administration of IB-MECA also results in significant preservation of ischemia-sensitive microtubule-associated protein 2 (Map-2), enhancement of the expression of glial fibrillary acidic protein (GFAP) and depression of nitric oxide synthase in ischemic brain tissue (von Lubitz 1999).

Summarizing the above discussed evidence, the protective/injurious effects of A_3AR during ischemia appear to depend on time after the onset of the ischemic insult which is consistent with the cascade of events described after ischemia (Dirnagl et al. 1999), some of which may also relate to inflamation. Initially, massive excitoxicity may be controlled by A_3AR and later, an inflammatory cascade could be potentiated by prolonged A_3AR stimulation. Timing of treatment with respect to the onset of the ischemic insult may therefore account for the different effects of pre- versus post-ischemia administration of A_3AR agonists. Moreover, A_3AR desensitization

may account for the different effects of acute versus chronic agonist treatments reported in different studies.

These observations raise the question of the time-related utility of A_3AR antagonists/agonists for treatment of ischemia. It may be speculated that after ischemia, prolonged treatment with A_3AR agonists first protects by reducing glutamate-mediated excitotoxicity thus supporting a depression of neuronal activity and energy save; later after ischemia because it desensitizes A_3AR, avoiding late onset deleterious A_3AR influences. This last-described protective effect could also be ascribed to antagonists administered late with respect to the onset of ischemia. However, the fact that preischemic stimulation of A_3AR in the in vivo model of global forebrain ischemia in the gerbil aggravates cerebral damage (von Lubitz et al. 1994) dictates caution in using A_3AR agonists to protection from ischemia-induced brain damage. Certainly further studies aimed at verifying the effect of agonists versus antagonists at different times after in vivo ischemia will help clarify the utility of this class of drugs in ischemia.

9.4.2 A_3AR and Neuroinflammation

The evidence summarized in the previous section suggests that A_3AR may control ischemic brain injury by controlling excitoxicity. Although excitotoxicity is invoked in the pathophysiology of most neurodegenerative central diseases, how A_3AR directly contribute to modulation of brain injury is largely unknown. Under neurodegenerative conditions involving ischemia, trauma, excitoxicity and bioenergetic dysfunctions, the interplay of resident glial cells with infiltrating peripheral bone marrow-derived cells produces neuroinflammation. An important role for A_3AR in modifying the inflammatory response was pointed out by first studies in mast cells where activation of these receptors appears to be responsible for release of allergic mediators contributing to inflammatory expansion (Ramkumar et al. 1993;Fozard et al. 1996; Gao et al. 2001) and mast cell degranulation (Reeves et al. 1997; Salvatore et al. 2000; Tilley et al. 2000; Zhong et al. 2003). On the other hand, exposure of blood peripheral cell lines to selective A_3AR agonists resulted in both anti- and proinflammatory effects (see: Gessi et al. 2008).

Microglia, astrocytes and oligodendrocytes are cell type sensors responding to neurodegenerative phenomena in the CNS. Evidence suggests that central A_3AR exert an important role in brain injury by affecting not only neurons but also glial function controlling important intracellular signaling pathways that are involved in neuroinflammation (Fig. 9.1). Below is a review of A_3AR influence on glial functions.

9.4.2.1 Effects of A_3AR in Astrocytes

A_3AR mRNA has been identified by Northern blot analysis in mouse astrocytes (Zhao et al. 1999). Early evidence indicates that A_3AR on astrocytes mediate both

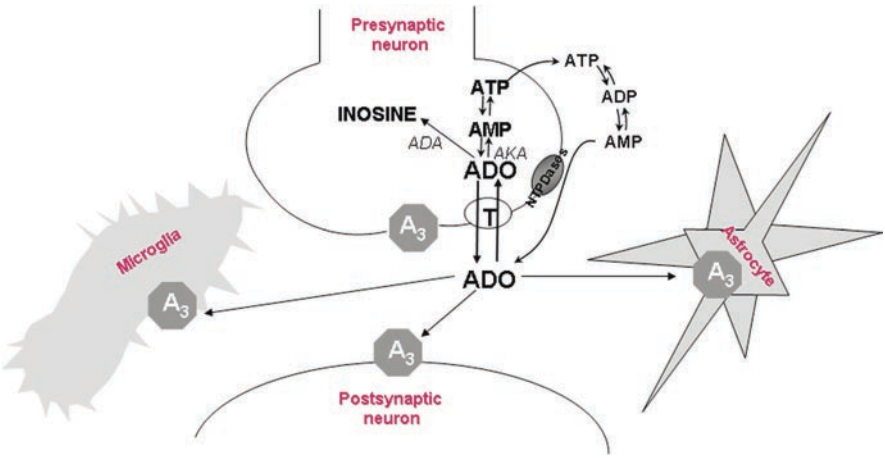

Fig. 9.1 Schematic diagram illustrating A_3 adenosine receptor localization in the brain. ADO: adenosine; ADA: adenosine deaminase; ATP: adenosine triphosphate, AMP: adenosine monophospate; AKA: adenosine kinase; T: bidirezional nucleoside transporter; NPTDase: family of ecto-nucleotidases, including NPTDase 1,2,3. During cerebral ischemia, extracellular ADO concentration increases acting on A_3 adenosine receptors located on different cell type

protection and cell death, depending on A_3AR agonist concentration (Abbracchio et al. 1997; Yao et al. 1997; Jacobson et al. 1999; Di Iorio et al. 2002).

On astroglial cell lines (human astrocytoma ADF cells) low (nM) concentrations of the selective A_3AR agonist Cl-IB-MECA induced a marked reorganization of cell cytoskeleton accompanied by induction of expression of small GTP-binding protein of the Rho family that is involved in control of actin cytoskeleton and by changes of intracellular distribution of the antiapoptotic protein Bcl-XL (Abbracchio et al. 1997, 2001).

In addition, stimulation of cultured murine astrocytes with Cl-IB-MECA induces the release of CCL-2, a chemokine which may exert neuroprotective effects (Wittendorp et al. 2004). Recently, in human D384 astrocytoma cells, Cl-IB-MECA at relatively low concentration (0.8 μM), reduced ATP depletion and apoptosis caused by hypoxic conditions. Furthermore, primary astrocytes prepared from A_3AR KO mice were more affected by hypoxia than those prepared from WT mice (Bjorklund et al. 2008b). In vivo, in the ischemia model of MCAo (transient, 30 min occlusions), IB-MECA administered after ischemia proved to decrease the intensity of reactive gliosis involving microglia and astrocytes as evaluated 7 days after ischemia. (von Lubitz et al. 2001). However some data have indicated no effect of A_3AR selective stimulation. In rat primary cultures, IB-MECA (1 μM) failed to modulate intracellular calcium signaling ($[Ca^{2+}]_i$) elicited by ATP (Alloisio et al. 2004) and no evidence was found that A_3AR affects intracellular calcium levels in acutely isolated rat astrocytes (Pilitsis and Kimelberg 1998).

Conversely, a high concentration (μM) of IB-MECA induced apoptosis of various cell lines including astrocytes (Yao et al. 1997; Jacobson et al. 1999). In agreement with this evidence, in primary cultures of rat astrocytes and in C6 glial cells, it was shown that treatment with the A_3AR agonist Cl-IB-MECA (10 μM) induced apoptosis and reduced the expression of endogenous Bcl-2, whereas it did not affect the expression of Bax. This suggests that intense activation of A_3AR is pro-apoptotic in glial cells via bcl2 and caspase-3 dependent pathways. (Appel et al. 2001). In primary cultures of mouse astrocytes, adenosine caused an increase in [$Ca^{(2+)}$i] most probably by acting on A_3AR (Chen et al. 2001). In rat cultured astrocytes, apoptosis caused by adenosine was significantly reduced by the selective A_3AR antagonist MRS1523 (Di Iorio et al. 2002).

On the whole, data are supportive that A_3AR may exert a cytoprotective or noxious effect in astrocytes, depending on the intensity of receptor stimulation.

9.4.2.2 Effects of A_3AR in Microglia

Several possible antiinflammatory effects are directly mediated by A_3AR on microglial cells. A_3AR mRNA is expressed in microglia (Fiebich et al. 1996) where it mediates several effects. Lee et al. (2006) have demonstrated that Cl-IB-MECA suppresses LPS-induced NF-kappaB activation and TNF-alpha production in mouse BV2 microglial cells. In primary mouse microglial cells and N13 microglia cell line, A_3AR stimulation increases both ERK1/2 and p38 MAPK phosphorylation via phosphatidylinositol-3′-kinase (Hammarberg et al. 2003, 2004). Interestingly, a concentration-dependent effect was noticed, that is high ERK1/2 phosphorylation occurred at low A_3AR agonist concentration, decreasing with increasing agonist concentration (Hammarberg et al. 2003).

Knowledge of the intracellular networks activated by adenosine A_3AR may help to elucidate the pathophysiological role of this receptor. Reactive gliosis, in response to central trauma, hypoxia/ischemia and neurodegeneration, includes phenotypical alterations of microglia and astrocytes and increased astrocyte number, occurs. Oligodendrocytes first encounter damage and death. After a central trauma, reactive gliosis is generally regarded as beneficial at first but, if prolonged, it may enhance tissue damage by production of deleterious factors (Neary and Snowden 1996). Glial A_3AR stimulated by high adenosine levels caused by a prolonged central trauma may well be implicated in neuroinflammatory tissue responses.

Plastic adjustments of A_3AR induced by brain injury might also be relevant to the modulation of intracellular pathways and cell safety. A_3AR appear to be very sensitive to prolonged stress in vitro. An up-regulation of A_3AR mRNA was observed 1 h after 3-nitropropionate exposure in hippocampal slices, normalization ensued 24 h later (von Arnim et al. 2000). A_3AR up-regulation was also reported in the hippocampus of a transgenic mouse model of Alzheimer's disease (APP23tg) where impaired oxidative phosphorylation was detected prior to amyloid deposition (von Arnim et al. 2006).

9.5 Conclusions and Perspectives

Adenosine A_3 receptors are widely distributed in the CNS but are expressed at a low level and have lower affinity for adenosine in comparison to the A_1 and A_{2A} receptors. Nevertheless, they appear to tonically modulate motor activity as pointed out in A_3AR-deleted mice. The role of A_3AR in several pathophysiological conditions is often controversial. In an attempt to synthesize the major evidence in the literature, it emerges that in conditions that create an extracellular increase of adenosine such as seizures or ischemia, A_3AR may contribute to neurotransmission and cell damage. Recently a pro-convulsant effect of A_3AR stimulation emerged, especially in the immature brain, thus raising the possibility that A_3AR might facilitate seizure-induced neuronal damage. This also raises the possibility that the A_3AR subtype plays a role in the establishment of activity-dependent plastic changes. Moreover, most data support a pro-nociceptive role of A_3AR involving both central nervous system and pro-inflammatory effects at peripheral tissues.

Major evidence for A_3AR in neurodegenerative phenomena emerges from studies performed in in vivo and in vitro models of hypoxia/ischemia. Data from the current literature suggest that the outcome of A_3AR stimulation on synaptic transmission during hypoxic/ischemic phenomena depends on the duration and intensity of the ischemic episode. It has been hypothesized that while A_3AR play a protective role in the first phase of ischemia by decreasing synaptic transmission, prolonged A_3AR stimulation by high adenosine concentrations could be pivotal in transforming the A_3AR-mediated effects from protective to injurious. Detrimental effects of A_3AR activation may be due, at least in part, to increased excitoxicity. Glial A_3AR stimulated by high adenosine levels caused by a prolonged central trauma may well be implicated in neuroinflammatory tissue responses. Moreover, A_3AR appear very sensitive to prolonged stress in vitro and plastic adjustments of A_3AR induced by brain injury and pharmacological treatment with agonists might be relevant to their final role. All these observations raise the question of the time-related utility of A_3AR antagonists/agonists for treatment of ischemia. Certainly further studies aimed at verifying the effect of agonists versus antagonists at different times after in vivo ischemia will help clarify the utility of this potent class of drugs in ischemia and in different neurodegenerative diseases.

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Chapter 10
Cardiovascular Biology of the A_3 Adenosine Receptor

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10.1 Cardiac Actions of the A_3AR

The varied effects of adenosine on electrical and mechanical properties of the heart were first reported in 1929 by Drury and Szent-Györgyi (Drury and Szent-Györgyi 1929). Adenosine slows heart rate, reduces atrial contractility, and slows conduction through the atrio-ventricular (AV) node (Belardinelli et al. 1989, 1995; Drury and Szent-Györgyi 1929; Shryock and Belardinelli 1997). Adenosine also suppresses the stimulatory effects of catecholamines mediated through β adrenergic receptors and inhibits the release of norepinephrine from sympathetic nerve fibers innervating the heart (Belardinelli et al. 1989, 1995; Dobson 1978; Schrader et al. 1977; Shryock and Belardinelli 1997). All of these actions of adenosine are generally protective in nature and serve to shelter the heart during conditions of inadequate blood flow or during increased cardiac work (Auchampach and Bolli 1999; Ely and Berne 1992).

Most if not all of the actions of adenosine on the heart are mediated by the G_i protein-coupled A_1 adenosine receptor (Belardinelli et al. 1989, 1995; Kurachi et al. 1986; Shryock and Belardinelli 1997). In atrial tissues, stimulation of the A_1 adenosine receptor activates an inward-rectifying potassium current named $I_{K\text{-}ado}$ that hyperpolarizes the cell membrane causing shortening of action potential duration (Belardinelli et al. 1989, 1995; Shryock and Belardinelli 1997). This is the same current activated by acetycholine and is regulated by pertussis toxin-sensitive G_i inhibitory proteins (Kurachi et al. 1986). Increased $I_{K\text{-}ado}$ via activation of the A_1 receptor is the underlying mechanism by which adenosine reduces heart rate and A-V nodal conduction and also explains the ability of adenosine to reduce contractile activity in atrial muscle

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(Belardinelli et al. 1989, 1995; Shryock and Belardinelli 1997). The depressant effects of adenosine on A-V nodal cells through activation of A_1 receptors and $I_{K\text{-}ado}$ explains the clinical efficacy of adenosine to terminate certain forms of supraventricular arrhythmias (DiMarco et al. 1983, 1984, 1990).

Activation of the A_1 receptor is also responsible for the anti-adrenergic actions of adenosine observed in both atrial and ventricular tissue (Belardinelli et al. 1989, 1995; Shryock and Belardinelli 1997). The anti-adrenergic actions of adenosine are largely explained by an inhibition of adenylyl cyclase activity and a decrease in the intracellular concentration of cAMP (Belardinelli et al. 1989, 1995; Dobson 1978; Hosey et al. 1984; LaMonica et al. 1985; Schrader et al. 1977; Shryock and Belardinelli 1997), although other signalling pathways including protein kinase C may contribute (Miyazaki et al. 2004). Activation of the A_1 receptor attenuates catecholamine-induced activation of $I_{Ca\text{-}L}$ (Isenberg and Belardinelli 1984) and potentially the delayed rectifier K^+ current I_K (Song et al. 1994). The anti-adrenergic actions are referred to as the "indirect" actions of adenosine whereas the responses observed in atrial tissues to activate $I_{K\text{-}ado}$ are referred to as "direct" actions of adenosine. In most species, adenosine exerts no direct actions on ventricular tissues (Belardinelli et al. 1989, 1995; Shryock and Belardinelli 1997; Song and Belardinelli 1996).

While most actions have been attributed to the A_1 receptor, there is increasing evidence that additional receptor subtypes may contribute to the effects of adenosine on the heart. Dobson and colleagues (Norton et al. 1999; Tikh et al. 2006; Woodiwiss et al. 1999) have promoted that the G_s protein-coupled A_{2A} receptor is expressed in cardiac muscle and modulates the anti-adrenergic actions of adenosine. Activation of the A_{2A} receptor appears to counteract the anti-adrenergic actions mediated via the A_1 receptor, through modification of cAMP levels (Norton et al. 1999; Tikh et al. 2006; Woodiwiss et al. 1999). Under conditions of poor coronary perfusion or ischemia when interstitial concentrations of adenosine increase markedly, suppression of the anti-adrenergic actions of adenosine may help to maintain cardiac performance. This action of the A_{2A} receptor may become even more prevalent during systemic sepsis since expression of the A_{2A} receptor is induced following exposure to pro-inflammatory mediators (Murphree et al. 2005; van der Hoeven et al. 2008). Additional studies have suggested that the A_{2B} receptor, coupled to pro-survival signalling pathways, may also be expressed in cardiac muscle and participate in the mechanism of ischemic preconditioning (Kuno et al. 2007; Eckle et al. 2007). This has yet to be firmly established and awaits further investigation.

Whether the A_3 receptor contributes to the regulatory action of adenosine in the heart appears unlikely. Administration of potent agonists of the A_3 receptor including IB-MECA, Cl-IB-MECA and CP-532,903 produce no hemodynamic effects when administered to experimental animals or humans[1] (Auchampach et al. 1997b, Auchampach et al. 2003; Kodani et al. 2001a; Takano et al. 2001; Tracey et al. 2003). Moreover, A_3 adenosine receptor agonists do not influence heart rate, cardiac

[1]Note that A_3 adenosine receptor agonists produce hypotension in rodents due to the actions of vasoactive mediators released from mast cells. This topic is discussed further in later sections of the book chapter.

performance, or coronary blood flow in isolated, buffer-perfused rodent hearts (de Jonge et al. 2002; Ge et al. 2006; Harrison et al. 2002; Peart et al. 2003; Peart et al. 2002; Tracey et al. 1997; Tracey et al. 2003; Wan et al. 2008). Thus, most evidence indicates that the A_3 receptor likely does not contribute to the regulatory actions of adenosine on the heart under normal conditions. However, detailed analysis of the potential actions of the A_3 receptor in cardiac muscle have not been conducted using newer agonist and antagonist ligands with greater selectivity for the A_3 receptor. Considering that it is coupled to inhibition of adenylyl cyclase like the A_1 receptor, it is plausible that the A_3 receptor also contributes to the anti-adrenergic action of adenosine in the ventricle. The question of whether or not functional A_3 receptor protein is expressed in cardiac muscle remains to be determined conclusively (see below).

10.2 Vascular Responses to the A_3AR

The original work of Drury and Drury and Szent-Györgyi (1929) also demonstrated that adenosine and adenine nucleotides possess vasoregulatory properties. Subsequent work detailed adenosine-dependent coronary vasodilatation in multiple species, and in 1963 the 'adenosine hypothesis' of coronary vasoregulation was formulated (Berne 1963; Gerlach et al. 1963). The precise role of endogenous adenosine in coronary control is still debated, with evidence both for and against involvement in regulating basal coronary tone or hyperaemic responses to (patho) physiological stimuli (Duncker and Bache 2008).

(1) A_3AR-Mediated Vasoregulation: The $A_{2A/2B}$ receptor sub-types are conventionally attributed with vasodilatatory functions in coronary vessels – the A_{2A} in mouse (Flood and Headrick 2001), pig (Hein et al. 1999), guinea pig (Belardinelli et al. 1998), and human vessels (Sato et al. 2005), and the A_{2B} in rat (Hinschen et al. 2003) and also humans (Kemp and Cocks 1999). These receptor sub-types are expressed in smooth muscle and endothelium, with evidence for both endothelium-dependent and independent components to adenosine-mediated dilatation (Headrick and Berne 1990; Rose'Meyer and Hope 1990). The A_1AR has also been attributed with vasoactive properties, mediating coronary constriction and/or countering dilatation via A_2ARs (Sato et al. 2005; Tawfik et al. 2005) or other stimuli (Hussain and Mustafa 1995). These interactions are consistent with co-expression of A_1, A_{2A} and A_{2B} receptors in coronary vessels (Wang et al. 2005), indicating that vascular control may ultimately be achieved via interplay between multiple receptor sub-types (and their effectors). Indeed, St Hilaire et al. (2009) suggest that shifts in adenosine receptor sub-type expression within a cell are key to induction of specific adenosine-mediated responses.

The vascular functions of the A_3 are perhaps least well understood of the adenosine receptor family, exacerbated by incomplete characterisation of cellular localisation of the A_3 receptor. There is some molecular evidence of A_3AR expression in rat aortic

smooth muscle (Zhao et al. 1997), while pharmacological evidence for vascular expression (based on dilatation to the partially selective agonist APNEA) has been acquired in guinea pig coronary vessels (Rubio and Ceballos 2003). The receptor is also expressed in mast cells that may reside within the vascular wall, where they may modify tone. The work of Wang and colleagues (2005) supports mRNA expression for all four receptor sub-types in coronary venules or arterioles in the pig, with expression of protein for all but the A_3AR. Thus, vascular effects of the A_3AR may well involve indirect signalling through non-vascular cell types such as mast cells (Shepherd et al. 1996).

The A_3AR exerts species-dependent effects on mast cell function, triggering degranulation in rodents (Ramkumar et al. 1993; Salvatore et al. 2000), but not dogs (Auchampach et al. 1997a). The receptor sub-type mediating degranulation of human mast cells is not clear, although the A_{2B} is functional in human tumour mast cells (Feoktistov and Biaggioni 1995). Within a species multiple mast cell types are targeted and similar signalling is involved – in mice A_3 receptor agonism increases histamine release from bone marrow-derived, cutaneous, and lung mast cells through G_i protein and phosphoinositide 3-kinase (PI3-K) dependent pathways (Laffargue et al. 2002; Tilley et al. 2000; Zhong et al. 2003). Duling and colleagues have demonstrated a role for mast cell activation in A_3-triggered vasoconstriction of peripheral vessels, via the release of factors such as histamine and thromboxane (Shepherd et al. 1996). Not only adenosine but also inosine (generated with adenosine deamination) binds to mast cell A_3ARs to trigger this degranulation, subsequently modifying vascular tone (Jin et al. 1997).

Additional support for a vasoconstrictor response to the A_3 receptor includes A_3AR dependent reductions in cAMP and adenylyl cyclase activity in smooth muscle, and inhibition of cAMP accumulation in cultured aorta (Zhao et al. 1997). Genetic deletion of the A_3AR elevates cAMP in heart and aortic tissue, and augments adenosine-mediated changes in tissue cAMP and blood pressure (Zhao et al. 2000). Talukder et al. (2002) report augmented A_{2A}AR-mediated vasodilatation in response to A_3AR knockout (or A_3AR antagonism in wild-type hearts). These findings collectively support an inhibitory function of the A_3AR, limiting cAMP-dependent responses and A_2AR-mediated vasodilatation. Given lower sensitivity of A_3 vs. A_{2A} receptors to adenosine, this could act to suppress excess vasorelaxation at high adenosine levels (such as those achieved during sepsis).

In contrast to A_3AR-dependent constriction, a small number of studies present evidence of A_3AR-mediated vasodilatation in rodents. Hinschen et al. (2003) reported potential A_3-mediated dilatation in rat hearts, based on blockade of responses to partially A_3-selective APNEA with the A_3AR antagonist MRS1191. Rubio and Ceballos (2003) identified coronary dilatation in response to APNEA in guinea pig hearts, though they did not further confirm the identity of the receptor(s) involved. Subsequently, Jenner and Rose'Meyer (2006) acquired evidence of concentration-dependent coronary relaxation with the A_3 agonist Cl-IB-MECA in rat heart, a response inhibited by MRS1191. Talukder et al. (2002) also observed coronary dilatation in response to high non-selective levels of Cl-IB-MECA in mouse hearts. In contrast, Lasley and colleagues report that the dilatatory effects of A_3AR agonists

in rat heart occur via A_{2A}ARs (Lasley et al. 1999), while there was no such response to A_3 agonism in the rabbit. Other studies report a lack of effect of A_3AR agonism on coronary tone in guinea pig (Maddock et al. 2002b) or mouse (Willems and Headrick 2007). The available, though variable, data suggests mixed vascular effects of A_3 agonism, with mediation of contraction (indirectly via mast cells, or via inhibition of dilatatory responses) or relaxation in different vascular beds and/or different species.

(2) A_3AR-Mediated Vascular Protection: Recent data support a vascular protective action of A_3 receptors (Zatta et al. 2006). Such an effect may not only limit vascular dysfunction, but could also contribute to limitations in infarct size and stunning in post-ischaemic myocardium (see A_3AR-Dependent Angiogenesis). Preservation of post-ischaemic coronary regulation following A_3AR agonism in guinea pig hearts (Giannella et al. 1997) provided initial evidence of A_3-mediated vasoprotection. A subsequent study confirmed A_3-dependent inhibition of vascular dysfunction in post-ischaemic mouse hearts (Zatta et al. 2006). Mechanisms underlying this vascular protection are not well understood, but may involve PKC and mito K_{ATP} channel activation (Maczewski and Beresewicz 1998). Recent findings also indirectly support modulation of ion (Na^+, H^+, Ca^{2+}) handling in this response (Zatta et al. 2006). Since the A_3AR can limit post-ischaemic or post-hypoxic apoptosis in other cell types (Chen et al. 2006; Das et al. 2005; De Jonge et al. 2002; Maddock et al. 2002a), it is feasible the A_3AR also limits post-ischaemic endothelial apoptosis (Scarabelli et al. 2001) to improve vascular control. There is also indirect evidence the A_3AR can enhance cellular anti-oxidant capacity (Maggirwar et al. 1994), which would additionally contribute to vasoprotection. Coronary vascular protection is apparent with exogenously applied A_3 agonist, but this may not reflect a normally harnessed response since A_3 antagonism alone is without effect on vascular outcomes (Zatta et al. 2006). Nonetheless, data support a beneficial effect of A_3 agonism on coronary dysfunction characteristic of the post-ischaemic heart.

(3) A_3AR-Mediated Vascular Damage and Permeability Changes: In contrast to adaptive or beneficial vascular responses, Platts and Duling (2004) have identified A_3-dependent modulation of the endothelial glycocalyx, which may be an important component of microvascular dysregulation. This matrix, on the luminal surface of all vessels, may be involved in regulating vessel permeability, leukocyte adhesion, and blood flow, and glycocalyx damage may underlie vascular abnormalities with ischaemia–reperfusion or inflammation (Platts et al. 2003). Activation of the A_3AR increases vascular permeability, and these effects of adenosine are mast cell dependent (Tilley et al. 2000). Thus, modulation of glycocalyx function, which dictates endothelial permeability and function, may occur indirectly via mast cell factors. High accumulation of adenosine (and inosine) with inflammation or ischaemia may trigger reductions in endothelial glycocalyx function via A_3 receptors, contributing to vascular dysfunction associated with these conditions. Again, here in the context of the vasculature, the A_3 receptor may mediate either beneficial or detrimental effects depending upon the extent and location of A_3 receptor engagement.

(4) A_3AR-Dependent Angiogenesis: Additional to potential vascular regulatory functions, the A_3AR may stimulate vascular growth. Adenosinergic control of angiogenesis was first evidenced by increased cardiac capillary density in response to adenosine in rabbits (Ziada et al. 1984). Adenosine-dependent increases in vascular density in avian tissue were documented shortly afterwards (Dusseau et al. 1986), and since these early studies investigators have confirmed 'direct' pro-angiogenic responses to A_{2A} and A_{2B} receptors, which promote endothelial proliferation and VEGF release (Grant et al. 1999; Takagi et al. 1996). 'Indirect' modulatory effects involve release of pro-angiogenic factors from vascular or inflammatory cells in response to activation of A_1 (Clark et al. 2007), A_{2A} (Leibovich et al. 2002), or A_{2B} and A_3 receptors (Feoktistov et al. 2003). The latter study suggests that A_3ARs act co-operatively with A_{2B} receptors to promote angiogenesis via differential expression of angiogenic factors in mast cells (angiopoietin-2 via A_3ARs, VEGF and IL-8 via A_{2B}ARs). The mast cell is known to participate in angiogenesis (Meininger and Zetter 1992), and it is well established that their function is A_3AR sensitive. Other more recent work confirms A_3AR-dependent stimulation of HIF-1α and VEGF expression, via endogenous or exogenous agonist, in hypoxic human cancer cells (Merighi et al. 2007). Overall, evidence suggests the A_3AR acts in a paracrine manner to enhance angiogenesis through triggering expression/secretion of factors such as VEGF, HIF-1α, and angiopoietin from non-vascular cell types.

10.3 Cardioprotective Actions of the A_3AR

The precise role of the A_3AR in cytoprotection has been clouded by observations of pro- and anti-apoptotic responses to A_3 agonism. This contributes to the view of the A_3AR as a somewhat enigmatic player in cell control (Gessi et al. 2008). The A_3AR may promote or limit cell death under specific conditions, and may promote or limit different aspects of inflammation. In terms of harnessing the receptor therapeutically, it may thus be considered a double-edged sword.

Despite evidence of minimal cardiac expression, pharmacological evidence supports A_3AR-dependent cardioprotection in ischaemic-reperfused myocardium from multiple species (Armstrong and Ganote 1994; Auchampach et al. 1997b, 2003; de Jonge et al. 2002; Gardner et al. 2004; Ge et al. 2006; Harrison et al. 2002; Hochhauser et al. 2007; Parsons et al. 2000; Peart et al. 2003; Peart et al. 2002; Tracey et al. 1997; Safran et al. 2001) including man (Carr et al. 1997). In terms of pharmacological protection from ischaemia-reperfusion, this can be achieved by pre-, intra-, or post-ischaemic A_3AR agonism. Augmented A_3 expression also enhances myocardial recovery from ischaemia or hypoxia (Black et al. 2002; Cross et al. 2002; Dougherty et al. 1998). These varied studies provide strong support for an A_3-dependent cardioprotective response, limiting cell death and enhancing functional outcomes in mammalian myocardium. Nonetheless, A_3AR agonism can also facilitate programmed cell death under certain conditions (Gessi et al. 2008),

and initial studies of A_3 gene deletion generated conflicting results supporting injury via A_3ARs (Cerniway et al. 2001; Harrison et al. 2002). Complicating matters, there is little direct evidence of A_3AR expression in cardiac tissue, with levels in murine myocardium at or below limits of detection via radioligand binding or Northern blot analysis (Black et al. 2002). Our own analysis of mRNA for the 4 receptor sub-types indicates that the A_3 has the lowest levels of transcript in murine myocardium or atrial-derived HL-1 cells (data not shown).

The cardioprotective function of intrinsically activated A_3ARs is contentious, although associations between variants of the A_1 and A_3 receptor genes and infarct size in patients with ischaemic cardiomyopathy support the importance of A_3 (or A_1) receptors to post-ischaemic outcome in humans (Tang et al. 2007). Initial studies of A_3AR deletion in mice actually identified increased resistance to ischaemic injury in the absence of the receptor (Cerniway et al. 2001; Guo et al. 2001; Harrison et al. 2002). These findings directly contrasted the effects of selective A_3AR agonists in wild-type hearts (Harrison et al. 2002). A suggestion that these paradoxical effects might originate in the models mixed genetic background (Harrison et al. 2002) was subsequently borne out by elimination of phenotypic differences in mice back-crossed for >12 generations (Ge et al. 2006). In these animals there is no discernible difference in ischaemic tolerance compared with wild-types. This is consistent with the view that cardiac A_3ARs are not normally engaged by endogenous adenosine to protect the heart, but are amenable to pharmacological activation by exogenous agonists. This raises the question of the linkage between A_3 receptor variants and infarct size in cardiomyopathic patients, which suggests that in the setting of this disease the receptor does play a role in dictating cardiac outcome (Tang et al. 2007). In addition to species differences, it may be that an intrinsic cardioprotective function of the A_3AR is only apparent under conditions of sustained or chronic disease such as ischemic cardiomyopathy, whilst not being apparent in otherwise healthy A_3AR knockout models.

The question of cellular location of the A_3AR in heart remains unresolved. Certainly the findings of Black et al. (2002) support a potent response to lowly expressed A_3ARs, with a steep gene-dosage effect: cardiac expression of only 12 fmol/mg of receptor confers substantial resistance to ischaemic insult, while only a fivefold increase in expression leads to cardiomyopathy. It is thus feasible that lowly expressed A_3 receptor protein is coupled with remarkable efficiency to downstream effectors. In terms of location, the ability of A_3AR agonists to protect isolated myocytes (Armstrong and Ganote 1994; Chaudary et al. 2004; Lee et al. 2001; Parsons et al. 2000) argues for myocyte expression and a direct myocardial response. Similarly, protection in cardiac preparations devoid of blood (Harrison et al. 2002; Maddock et al. 2002a, 2003; Peart et al. 2002) supports mediation of protection independently of circulating inflammatory cells, though a role for residual cells within the cardiac wall cannot be excluded. However, the recent work of Ge et al. (2006) provides convincing evidence that the protective effects of A_3AR agonism involve direct cardiac responses independent of mast cells.

A variety of end-points evidence A_3-dependent cardioprotection, including reductions in infarct size (Auchampach et al. 1997b, 2003; Maddock et al. 2002a;

Tracey et al. 2003), inhibition of apoptosis (Maddock et al. 2002a), and improvements in post-ischaemic contractile function (Gardner et al., 2004; Maddock et al. 2003). These salutary actions have been documented in cardiac tissue from multiple species including mice (Harrison et al. 2002; Peart et al. 2002), rats (Hochhauser et al. 2007; Maddock et al. 2002a; Safran et al. 2001), guinea pigs (Maddock et al. 2003), rabbits (Armstrong and Ganote 1994; Auchampach et al. 1997b; Tracey et al. 1997, 1998, 2003), chickens (Parsons et al. 2000), dogs (Auchampach et al. 2003), and humans (Carr et al. 1997).

(1) Relevance of Timing of A_3AR Agonism: As already noted, A_3AR activation can exert protection at differing times prior to, during or after ischaemia. A number of investigations confirm that treatment with A_3AR agonist prior to or during ischaemia/hypoxia confers benefit in in vitro or in vivo models, reducing cell death and enhancing functional outcomes (Auchampach et al. 1997b, 2003; Gardner et al. 2004; Ge et al. 2006; Harrison et al. 2002; Peart et al. 2003; Peart et al. 2002; Safran et al. 2001). In addition, transient pre-ischaemic activation of A_3ARs can confer preconditioning effects in the heart. Though some studies, such as those of Thourani et al. (1999a, b) and Chaudary et al. (2004), purport assessment of preconditioning responses, the receptor agonists are present in these models up to (and thus during) the ischaemic episode, without any intervening washout. Indeed, in vivo studies in which A_3 agonist is applied prior to ischaemia do not address preconditioning, as the agents may remain for extended periods within the myocardial extracellular space to activate receptors at later time points. These studies thus do not evidence preconditioning per se, but rather provide further support for benefit with combined pre- and intra-ischaemic A_3AR activation.

The early work of Armstrong and Ganote (1994) did reveal A_3-dependent preconditioning effects in isolated cardiomyocytes. Subsequent studies report preconditioning with transient pre-ischaemic A_3AR agonism in isolated cells (Parsons et al. 2000; Lee et al. 2001), intact hearts (de Jonge et al. 2002; Hochhauser et al. 2007; Tracey et al. 1997, 1998, 2003) and human myocardial tissue (Carr et al. 1997). Transient activation of the A_3AR therefore elicits a protective response persisting after stimulus withdrawal. In addition to acute protection, A_3AR pre-treatment can also elicit a delayed or second window of protection (Kodani et al. 2001b; Takano et al. 2001; Zhao and Kukreja 2002). Interestingly, preconditioning responses to other stimuli may also involve the A_3AR. For example, A_3ARs are implicated in preconditioning with resveratrol (Das et al. 2005), involving CREB- and PI3K/Akt-dependent regulation of Bcl-2.

Cardioprotection via A_3ARs can also be triggered post-ischaemia – the study of Jordan et al. (1999) indicates that A_3AR activation on reperfusion limits neutrophil-dependent injury, while Maddock et al. (2002a, 2003) showed a reduction in myocardial apoptosis following A_3AR agonism during reperfusion. Post-ischaemic protection is also supported by work by Auchampach et al. (2003), who showed effective reduction of infarct size with either pre- or post-ischaemic A_3AR agonism. These postconditioning-like effects of the A_3AR may be of greater clinical relevance

than preconditioning effects, given the unpredictability of ischaemic onset except in cases of planned surgery. Postconditioning describes cardioprotection via transient ischaemia or other stimuli during the early reperfusion phase following the prolonged ischaemic insult (Zhao et al. 2003). Kin et al. (2005) presented evidence of a role for the A_3AR in protection with ischaemic postconditioning. Subsequent work, however, has identified essential roles for the A_1 (Xi et al. 2008), A_{2A} (Morrison et al. 2007), or A_{2B} receptor (Philipp et al. 2006) in postconditioning, but not for the A_3AR.

(2) Effectors of A_3AR-Dependent Protection: With respect to signalling, the early work of Liang and colleagues supported a RhoA-phospholipase D interaction in A_3-mediated cardioprotection (Parsons et al. 2000; Lee et al. 2001), although this stems from work in avian and not mammalian cells. Protective responses to the A_3AR in myocardium are typically attributed to mitochondrial K_{ATP} channel activation (Peart et al. 2003; Thourani et al. 1999a; Tracey et al. 1998), with several kinase signalling pathways implicated in transducing signal from the sarcolemmal receptors, including Erk1/2 (Germack and Dickenson 2004), PKC (Peart et al. 2003), and PI3K/Akt (Germack et al. 2004). However, though A_3ARs may couple to PI3K and Erk1/2 signalling, it is important to note that these proteins may not necessarily play any role in associated cardioprotection (Button et al. 2005). The A_3AR can also regulate MAPK signalling (Schulte and Fredholm 2003), and Hochhauser et al. (2007) recently reported on a contribution of p38 MAPK to A_3AR mediated cardioprotection.

Interestingly, the recent study of Wan et al. (2008) indicates that preconditioning with A_3 agonism or transient ischaemia is entirely dependent upon functional sarcolemmal K_{ATP} channels, being abrogated by deletion of the pore-forming component of the channel. Moreover, this study provided direct evidence, using electrophysiological techniques, for expression of A_3ARs coupled to K_{ATP} channel activation in mouse ventricular cardiomyocytes (Fig. 10.1; Wan et al. 2008). This fuels ongoing controversy regarding the identity of the K_{ATP} channels involved in protective responses to preconditioning or other stimuli (Gross and Peart 2003). The mitochondrial rather than the sarcolemmal K_{ATP} channel is more commonly linked to A_3AR-dependent protection. Interestingly, recent work adds further support to cardioprotection via the sarcolemmal K_{ATP} channels, and a link between these channels and mitochondrial function and damage (Marinovic et al. 2008). Thus, a more complex interplay may exist between sarcolemmal signal components and the mitochondria. Within mitochondria the permeability transition pore is considered a major target for protective responses including pre- and post-conditioning (Halestrap et al. 2004), and Hausenloy and colleagues presented initial evidence that adenosine receptor mediated cardioprotection (specifically A_1AR dependent) could be reduced by interfering with MPTP functionality (Hausenloy et al. 2004). Park et al. (2006) more recently showed that post-ischaemic protection with A_3 agonism in rats involves PI3-kinase/Akt-dependent inactivation of GSK-3β, with subsequent inhibition of the MPTP. Shneyvays et al. (2005) have shown inhibition of the dissipation of the mitochondrial membrane potential in myocytes treated with

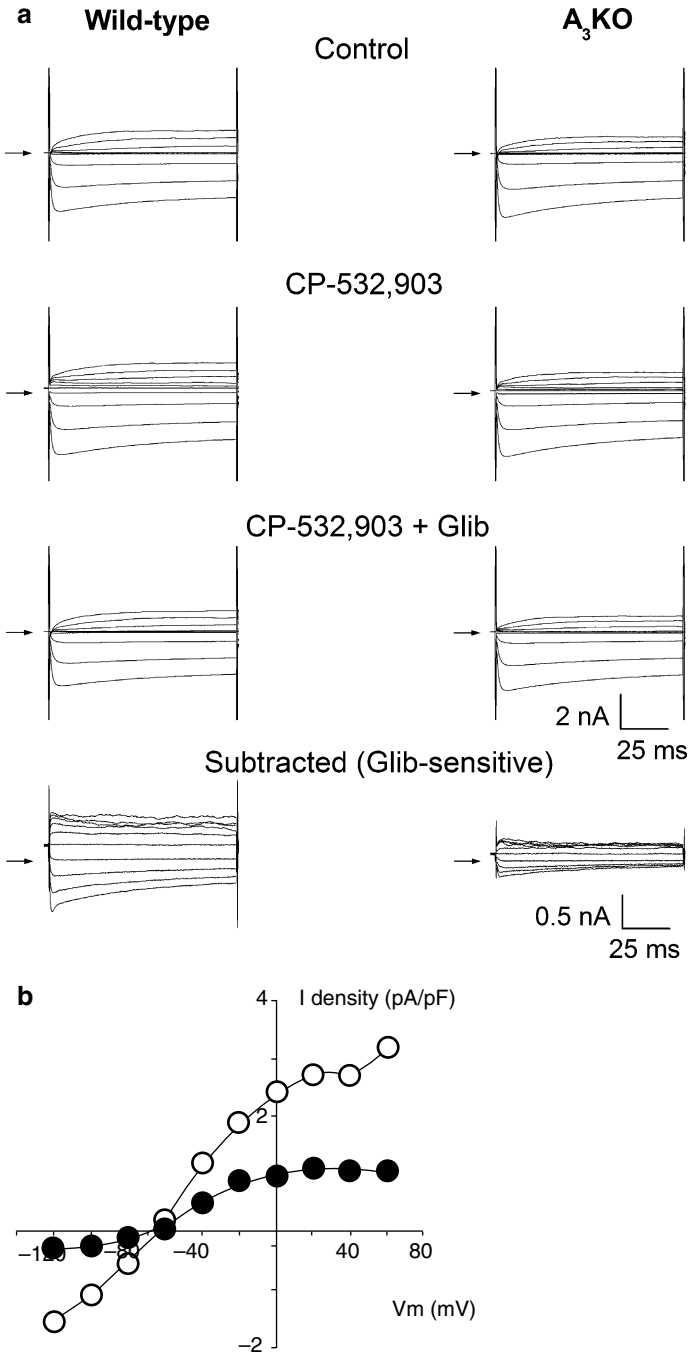

Fig. 10.1 Evidence for coupling between the A_3 adenosine receptor and opening of the K_{ATP} channel in isolated mouse ventricular cardiomyocytes. (*Panel* **a**) Whole-cell current traces recorded

A_3 agonist, consistent with an action on the MPTP. They also detected reductions in intracellular Ca^{2+} during hypoxia, which may both result from and/or contribute to improved mitochondrial function.

Beyond the MPTP a number of potential protective targets have been identified. The study of Zucchi et al. (2001) shows A_3AR-dependent reductions in sarcoplasmic reticulum Ca^{2+} channel density, akin to those observed with ischaemic preconditioning. There is indirect support for A_3AR mediated enhancement of anti-oxidant capacity in cardiac and vascular cells (Maggirwar et al. 1994). Interrogation of transgenic lines overexpressing the A_3AR supports modulation of intracellular energy metabolism, with preservation of ATP content during ischaemia (Cross et al. 2002). This agrees with known protective effects of adenosine on myocardial energy state during ischaemia (Fralix et al. 1993) or reperfusion (Angello et al. 1991).

Given the key role of inflammatory processes in determining infarct size and post-ischaemic outcomes, the anti-inflammatory actions of the A_3AR might be important in mediating cardioprotection in vivo. As outlined by Vinten-Johansen et al. (1999), adenosinergic protection via A_2 and A_3 receptor sub-types may involve both direct myocardial and indirect inflammatory blood cell dependent responses. Although in vitro studies confirm the ability of A_3ARs to more directly limit myocardial injury (Armstrong and Ganote 1994; Chaudary et al. 2004; Harrison et al. 2002; Maddock et al. 2002a; Parsons et al. 2000; Peart et al. 2002, 2003), and there appears to be no essential role for mast cells (Ge et al. 2006), inflammatory cells may be important in situ. For example, A_3AR agonism limits radical formation and chemotaxis of activated neutrophils (van der Hoeven et al. 2008). The study of Jordan et al. (1999) supports mediation of protection in vivo via modulation of neutrophil function post-ischaemia.

(3) Effects of Age and Disease: A concern regarding potential cardioprotective interventions is their efficacy in diseased or aged hearts. It is increasingly evident that responses to both ischaemia and protective stimuli differ in aged vs. young myocardium, with the older heart most likely to require anti-ischaemic therapy in humans (Peart and Headrick 2008). It has been shown that adenosinergic protection can be eliminated with aging (Headrick et al.

Fig. 10.1 (continued) from ventricular myocytes isolated from wild-type mice and from A_3 receptor gene knock-out mice in the presence of vehicle (Control), the A_3 receptor agonist CP-532,903 (1 μM), and in the presence of both CP-532,903 and the K_{ATP} channel antagonist glibenclamide (1 μM). The glibenclamide-sensitive current was obtained by digitally subtracting the current obtained in CP-532,903 + glibenclamide from that in CP-532,903 alone. (*Panel* **b**) Corresponding current-voltage relationship of the glibenclamide-sensitive current elicited by CP-532,903 in wild-type (*open circles*) and A_3 receptor KO (*closed circles*) myocytes. Current is depicted as current density (pA/pF) to normalize for cell size. In all experiments, recordings were obtained in the presence of the A_1 receptor antagonist CPX (500 nM) and the A_{2A} receptor antagonist ZM 241385 (500 nM) in the bathing solution and with 0.2–0.5 mM ATP in the recording pipette to "prime" opening of the K_{ATP} channel (Reproduced with permission from Wan et al. (2008). Copyright 2008, American Society for Pharmacology and Experimental Therapeutics.)

2003), as can responses to other preconditioning (Schulman et al. 2001) or postconditioning stimuli (Przyklenk et al. 2008). Unfortunately, there is no information regarding the ability of A_3AR agonists to attenuate injury in the aged heart. However, we have shown deterioration of adenosine-triggered cardioprotection (Headrick et al. 2003), a response that involves an A_3-dependent component (Peart et al. 2003). Based on these observations one might surmise a decline in protective efficacy of the A_3AR with age, though this requires further and more direct testing.

There is evidence that cardioprotective stimuli such as pre- or postconditioning may also be impaired by different pathologies relevant to heart disease, including diabetes (Tosaki et al. 1996; Kersten et al. 2000), obesity (Bouhidel et al. 2008), and hypertrophy (Miki et al. 2000; Ebrahim et al. 2007). Unfortunately, relatively little research has been undertaken into the impact of these diseases on protective A_3AR responses. There is evidence activation of A_3ARs retains cardioprotective efficacy in hypertrophic myocardium (Hochhauser et al. 2007), whereas there is evidence of failure in other protective responses in hypertrophic or remodelled hearts. There is also evidence that the A_3 may limit hypertrophic growth itself (Gan et al. 2005). On the other hand, Lu et al. (2008) present evidence that A_3ARs may actually exacerbate development and expression of pressure-overload hypertrophy. This latter study is interesting, as it suggests a pro-growth action of a receptor that is additionally attributed with pro-apoptotic actions. An effect of the A_3AR on cardiac growth is supported by the cardiomyopathic phenotype evident upon cardiac overexpression of the A_3AR (Black et al. 2002). In terms of clinical utility, it will be critical to develop further information regarding the efficacy of protective A_3 responses in diseased hearts, particularly with relevant disease states such as diabetes (where hypoglycaemic agents may also block K_{ATP} channel responses), hypertension and hypertrophy, and dyslipidemias that are known to influence other protective responses.

10.4 Summary and Future Directions

While the A_1 and A_{2A} receptors clearly play a more prominent role, current evidence suggests that the A_3 receptor contributes importantly to the cardiovascular actions of adenosine. The A_3 receptor appears to play a more prominent role under ischemic conditions and remains a promising target for promoting angiogenesis and treating disorders associated with acute ischemia. As clinical agents, A_3 receptor agonists show substantial promise considering that they can be administered at relatively high doses, even to humans (van Troostenburg et al. 2004), with minimal effect on systemic hemodynamic parameters. Future research is needed to further clarify mechanisms by which A_3 receptor activation protects against ischemic injury and to explore more extensively the importance of this receptor subtype in the pathogenesis of additional cardiovascular diseases and aging.

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Chapter 11
A_3 Adenosine Receptor in the Pulmonary System

Yifat Klein and Idit Matot

11.1 The Expression Pattern of A_3 Adenosine Receptor in the Lung: Interspecies Differences and Functional Implications

The A_3 adenosine receptor (A_3AR) has been cloned from multiple species including rat (Meyerhof et al. 1991; Zhou et al. 1992), sheep (Linden et al. 1993), canine (Auchampach et al. 1997), rabbit (Hill et al. 1997), mouse (Zhao et al. 1999), chick (Durand and Green 2001), and human (Salvatore et al. 1993; Sajjadi and Firestein 1993).

A_3AR has a widely distributed expression pattern, its mRNA being detected in the heart, brain, proximal colon, testes, lungs, kidneys, placenta, spleen, liver, uterus, bladder, jejunum, and eye (Zhou et al. 1992; Salvatore et al. 1993; Rivkees 1994; Linden 1994; Dixon et al. 1996). However, marked differences exist in expression levels within and among species.

High levels of A_3AR expression were found on murine lung mast cells, where it was shown to mediate pro-inflammatory effects (Salvatore et al. 2000). Both in vitro (Ramkumar et al. 1993; Thorne et al. 1996) and in vivo (Shepherd et al. 1996; Fozard et al. 1996) studies in a variety of rodent species have established that activation of A_3AR results in mast cell degranulation and/or enhancement of allergen-induced mediator release (Ramkumar et al. 1993; Fozard et al. 1996; Reeves et al. 1997; Jin et al. 1997; Zhong et al. 2003). In human, however, A_3AR was not found in association with lung mast cells (Walker et al. 1997), thus decreasing the relevance of these animal models.

The human A_3AR exhibits a unique expression pattern, which differs from those of its homologues, as well as from the other human adenosine receptor (AR)

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subtypes. Human A_3ARs are predominantly expressed in the lungs and liver, while low levels have been found in the aorta and brain (Salvatore et al. 1993; Sajjadi and Firestein 1993). Within the human lungs, A_3AR appears mostly on mesenchymal cells and eosinophils in the lamina propria of the airways, and in the adventitia of blood vessels (Bai et al. 1994; Walker et al. 1997), but not on mast cells. A_3AR is highly expressed on circulating and resident inflammatory cells within the lung, particularly granular leukocytes such as eosinophils, basophils, and neutrophils, as opposed to mononuclear cells which exhibit low to moderate expression (Linden 1994; Bai et al. 1994; Kohno et al. 1996; Walker et al. 1997; Knight et al. 1997; Gessi et al. 2002). There is now accumulating evidence that A_3AR activation modulates functional changes in these cells, as discussed in the next section.

With respect to other AR subtypes, binding studies in human healthy lung tissues have suggested that A_2 receptor subtype is much more abundant than the A_1 and A_3 receptor subtypes (Joad 1990). In addition, binding studies revealed that the A_1 and A_{2A} subtypes have the highest affinity for adenosine, whereas the A_{2B} and A_3 receptors have a significantly lower one (Fredholm et al. 2001b). As virtually all cells express one or more of the AR subtypes, the relative expression of the A_3AR and the other AR subtypes, which in many processes elicit opposing effects (Jacobson 1998; Polosa 2002; Caruso et al. 2006; Brown et al. 2008b), will eventually determine the cell response to adenosine.

11.2 A_3 Adenosine Receptor in Reactive and Inflammatory Diseases of the Airways: Functional Role and Therapeutic Applications

11.2.1 Pro and Anti-inflammatory Functions of Adenosine A_3 Receptor in the Inflamed Airway (Table 11.1)

The A_3AR has emerged as an AR subtype that may serve important regulatory roles in the inflamed airways. Walker et al. (1997) demonstrated that A_3AR transcript and protein levels are elevated in lung biopsies from patients with asthma or chronic obstructive pulmonary disease (COPD), suggesting increased signaling through this receptor. Moreover, numerous in vivo and ex vivo studies imply that A_3AR signaling can influence inflammatory cell types associated with asthma and COPD (Knight et al. 1997; Ezeamuzie and Philips 1999; Jatakanon et al. 1999; Salvatore et al. 2000; Reeves et al. 2000; Zhong et al. 2003; Ezeamuzie and Philips 2003). For example, in the human lung, A_3AR mediates inhibition of eosinophil migration induced by platelet activating factor (PAF), one of the most potent chemoattractants for eosinophils, or other soluble mediators (Walker et al. 1997; Knight et al. 1997). In addition, A_3AR suppresses two important inflammatory functions of human eosinophils: degranulation and oxygen free radical release (Ezeamuzie and Philips 1999; Reeves et al. 2000). It has also been demonstrated

Table 11.1 Pro- and anti-inflammatory effects of A_3AR activation on leukocytes

Cell type/species	Function	Reference
Human eosinophils	Inhibition of chemotaxis	Walker et al. 1997; Knight et al. 1997
	Inhibition of degranulation and Free radical release	Ezeamuzie and Philips 1999; Reeves et al. 2000; Ezeamuzie 2001
	Elevation of intracellular Ca^{2+}	Kohno et al. 1996
Human neutrophils	Inhibition of oxidative burst	Bouma et al. 1997; Gessi et al. 2002
	Inhibition of degranulation	Bouma et al. 1997
Human monocytes and macrophages	Inhibition of inflammatory cytokine production	Bouma et al. 1994; Le Vraux et al. 1993; McWhinney et al. 1996; Sajjadi et al. 1996
	Inhibition of respiratory burst	Broussas et al. 1999
Murine macrophages	Inhibition of inflammatory cytokine production and release	Bowlin et al. 1997; Szabó et al. 1998; Haskó et al. 1996, 1998
	Upregulation of anti-inflammatory cytokine production	Haskó et al. 1996
Murine mast cells	Degranulation and mast cell-mediated increase in cutaneous vasopermeability	Ramkumar et al. 1993; Ali et al. 1990; Fozard et al. 1996; Reeves et al. 1997; Tilley et al. 2000, 2003; Zhong et al. 2003
Murine neutrophils	Airway infiltration	Tilley et al. 2003
Guinea pig eosinophils and macrophages	Chemotaxis and airway infiltration	Spruntulis and Broadley 2001

that A_3AR mediates anti-inflammatory functions of human neutrophils and monocytes. Activation of A_3AR on neutrophils inhibits oxidative burst, degranulation and tumor necrosis factor α (TNFα) release (Bouma et al. 1994, 1997; Gessi et al. 2002). In monocytes and macrophages A_3AR activation inhibits superoxide anion generation, suppresses pro-inflammatory cytokine release, e.g. endotoxin-induced TNFα, interleukin-12 (IL-12), and interferon-γ, and upregulates anti-inflammatory cytokine production, e.g. IL-10 (Le Vraux et al. 1993; Sajjadi et al. 1996; McWhinney et al. 1996; Haskó et al. 1996; Bowlin et al. 1997; Szabó et al. 1998; Haskó et al. 1998; Broussas et al. 1999). Therefore, the presence of high levels of A_3AR in the asthmatic lung may represent a way for attenuation of eosinophilic- and neutrophilic-induced inflammation via A_3AR activation, suggesting its counter-inflammatory involvement in the pathophysiology of chronic inflammatory diseases of the airways.

Eosinophils are known to play a role in the pathophysiology of allergic diseases, especially asthma (Frigas and Gleich 1986; Barnes 1989). They infiltrate the

asthmatic lung, where they release tissue-damaging granular proteins such as the major basic protein, allergic mediators such as leukotrienes, as well as oxygen free radicals, which in concert orchestrate bronchial inflammation and consequently bronchial hyperresponsiveness (Silberstein 1995). Human eosinophils highly express the A_3AR (Kohno et al. 1996). The effect of A_3AR signaling on this cell type, however, remains controversial. As discussed thoroughly above, A_3AR mediates anti-inflammatory processes such as inhibition of eosinophil migration and degranulation. Nevertheless, pro-inflammatory activities have also been reported in some species (Walker 1996; Tilley et al. 2000; Spruntulis and Broadley 2001; Zhong et al. 2003; Fan et al. 2003; Young et al. 2004; Hua et al. 2008), and stimulatory effects have been observed in human eosinophils following A_3AR activation (Kohno et al. 1996). Kohno et al. (1996) reported A_3AR-mediated elevation of intracellular calcium and expression of activation markers in eosinophils obtained from subjects with hypereosinophilia. This finding is in agreement with the stimulatory role of this receptor subtype on murine mast cells, as reported by others (Ramkumar et al. 1993; Fozard et al. 1996; Reeves et al. 1997; Zhong et al. 2003). A_3AR-mediated pro-inflammatory effect was also observed in a guinea pig model of allergy, in which it caused rapid in vivo chemotaxis of eosinophils and macrophages into inflamed airways of sensitized animals (Spruntulis and Broadley 2001). The different pro- and anti-inflammatory effects of A_3AR activation on leukocytes are summarized in Table 11.1.

11.2.2 Mechanisms for the Pro and Anti-inflammatory Actions of Adenosine A_3 Receptor During Airway Inflammation

The opposing effects of the A_3AR elicited by A_3AR activation were allegedly to either altered receptor coupling or differences in A_3AR expression between eosinophils obtained from hypereosinophilic versus normal tissues. However, the A_3AR-mediated anti-inflammatory effect was apparent in eosinophils obtained from lungs of both healthy, non-smoking donors, and from subjects with inflammatory airway disorders (Walker et al. 1997), characterized by extensive infiltration with activated eosinophils (Gleich 1990; Polosa et al. 2000), suggesting that hypereosinophilia per se does not alter A_3AR response in this context.

The diverse responses to A_3AR activation, as documented in the literature and discussed in previous paragraphs, may be attributed to the different downstream signal transduction pathways which are cell type-, and G protein-dependent. A_3ARs may be coupled to either G_i or $G_{q/11}$ proteins, thereby either lower the intracellular cyclic adenosine monophosphate (cAMP) levels and affecting Ca^{2+} and K^+ channels or elevate intracellular second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), respectively (Ali et al. 1990; Zhou et al. 1992; Salvatore et al. 1993; Kohno et al. 1996; Hoffman et al. 1997; Fredholm et al. 2001a; Linden 2001). A_3ARs are believed to be negatively coupled to adenylate cyclase by virtue of their

ability to mediate inhibition of forskolin-induced generation of cAMP in Chinese hamster ovary cells stably transfected with the cloned receptors (Zhou et al. 1992; Jackson 1998; Englert et al. 2002) or transformed human cells (Gessi et al. 2001).

The reports that A_3ARs mediate mobilization of intracellular Ca^{2+} (Kohno et al. 1996) and inhibition of adenylate cyclase would appear to favour eosinophil stimulation, rather than inhibition. It is well known that a rise in intracellular calcium is an important requirement for eosinophil activation (Kita et al. 1991) and that cAMP is a powerful inhibitor of eosinophil responses (Van der Bruggen and Koenderman 1996). However, evidence proved otherwise, and in fact A_3AR activaion was shown to have an inhibitory effect on human eosinophils (Walker et al. 1997; Knight et al. 1997; Ezeamuzie and Philips 1999; Reeves et al. 2000; Ezeamuzie 2001), Chinese hamster ovary cells expressing cloned A_3ARs (Zhou et al. 1992; Jackson 1998), and even Jurkat T cells (immortalized line of T lymphocyte cells) (Gessi et al. 2001). This discrepancy has led researchers to investigate the coupling of A_3ARs to adenylate cyclase in human eosinophils, where they found that an atypical form of A_3ARs positively coupled to adenylate cyclase may exist (Ezeamuzie 2001; Ezeamuzie and Philips 2003). The resulting cAMP generation may underlie the anti-inflammatory actions of A_3 agonists in eosinophils.

A possible explanation for these observations may also be related to differences in A_3AR coupling in transfected or transformed cells as opposed to native cells. It is well known that expression/over-expression of cloned receptors often result in coupling that differs from, or sometimes is opposite to, that of naturally expressed receptors (Aune et al. 1993; Cowen et al. 1997; Neumann et al. 1999; Englert et al. 2002). Such variations probably reflect differences in G-protein involvement or adenylate cyclase isoform expression (Cooper et al. 1995). It is also possible that A_3ARs in eosinophils are atypical – perhaps representing a subclass of A_3ARs, or it may be that A_3ARs simply exhibiting cell-specific coupling, as has been previously noted for prostaglandin E receptors (Aarab et al. 1999).

Altogether, these data indicate that A_3AR exhibits potentially varying signaling capabilities and regulatory susceptibility, which might explain the dual role ascribed to this receptor in the literature.

11.2.3 Adenosine A_3 Receptor in Asthma, COPD and Allergic Rhinitis

In asthma and COPD patients, the concentration of adenosine in the bronchoalveolar lavage fluid and exhaled breath condensate is higher compared to normal patients (Huszar et al. 2002). Moreover, inhalation or intravenous administration of adenosine causes bronchoconstriction in these patients, but not in normal subjects (Cushley et al. 1984; Oosterhoff et al. 1993; Drake et al. 1994; Polosa et al. 2002). Since this effect could be significantly blocked by histamine and leukotriene antagonists, or agents that block mast cell degranulation, it is believed to be due to degranulation of mast

cells (Richards et al. 1988). Bronchospasm seems to result from the release of histamine, and additional mediators such as leukotrienes and arachidonic acid derivatives, which cause the contraction of smooth muscles (Polosa et al. 1995; Spicuzza et al. 2003). Accumulating data suggest that adenosine-induced bronchoconstriction in humans is mediated via A_{2B}AR, which is expressed on human mast cells, as well as smooth muscle cells, and has been shown to mediate pro-inflammatory events (Clarke et al. 1989; Feoktistov et al. 1998; Feoktistov and Biaggioni 1998; Ryzhov et al. 2004; Zhong et al. 2004; Holgate 2005). However, since A_1AR, an AR subtype which does not appear to be expressed on mast cells, has also been implicated in airway obstruction (Bjorck et al. 1992; Nyce and Metzger 1997; Abebe and Mustafa 1998; Brown et al. 2008a), and because mast cells are not an exclusive cellular source of bronchoconstrictive mediators, it is not possible to conclude that mast cells are the only mediators of bronchoconstriction in response to adenosine, alternative mechanisms should not be disregarded, e.g., direct effect on smooth muscle cells of the airways.

The relevance of the A_3AR to airway inflammation in human, is yet uncertain. Human A_3ARs are extensively expressed in the lungs (Salvatore et al. 1993) but appear to be present on eosinophils, rather than mast cells (Bai et al. 1994; Walker et al. 1997), thus questioning their contribution to the observed adenosine-induced bronchoconstriction. A_3AR inhibits eosinophil degranulation, and therefore it does not seem to be directly involved in the bronchocospastic process. What is clear, however, is that A_3AR is upregulated in the inflamed lung, predominantly on eosinophils (Walker et al. 1997; Varani et al. 2006). Moreover, in vitro studies have demonstrated that activation of A_3AR leads to the inhibition of eosinophil chemotaxis and activation, as well as anti-inflammatory functions of neutrophils and monocytes, suggesting a protective role for this receptor in reactive airway disease as discussed above.

The accumulation of evidence implicating a role for adenosine in the pathogenesis of airway inflammation has led to investigations into all adenosine receptor subtypes as potential therapeutic targets for the treatment of asthma and COPD. To this end, the efficacy of A_3AR in the clinical setting has been inconclusive. However, inhibition of A_1 and A_{2B} receptors and/or activation of the A_{2A} pathway may serve as an alternative therapeutic approach (Brown et al. 2008b).

As in asthma and COPD, mast cells are major players in the pathogenesis of inflammation in allergic rhinitis (Polosa et al. 1995, 1999; Ludviksdottir et al. 2000). Inflammation in allergic rhinitis is also characterized by eosinophilic infiltration into the airways. Studies evaluating the role of ARs in allergic rhinitis found that intranasal treatment with an A_{2A}AR agonist/A_3AR antagonist following intranasal allergen challenge had limited clinical benefits for these patients (Rimmer et al. 2007). The authors concluded that A_3ARs and A_{2A}ARs may play only a limited role in the pathogenesis of allergen-associated nasal inflammation. However, as previous data indicated that A_3AR may play an anti-inflammatory role in allergic inflammation of the airways, an alternative therapeutic approach may consist of local administration (inhalant) of a double A_{2A}AR/A_3AR agonist.

11.3 Adenosine A_3 Receptor and Lung Injury: Functional and Clinical Implications

11.3.1 Adenosine A_3 Receptor and Ischemia–Reperfusion-Induced Lung Injury

11.3.1.1 The Pathophysiology and molecular basis of Ischemia–Reperfusion-Induced Lung Injury

Acute lung injury secondary to ischemia–reperfusion (IR) is a major complication most commonly associated with lung transplantation (King et al. 2000; De Perrot et al. 2003), that leads to higher post-operative morbidity, mortality, and late complications including chronic rejection (Maxey et al. 2004). IR injury might also result from other thoracic surgery procedures, including cardiopulmonary bypass, pulmonary thromboendarterectomy or thrombolysis, and pulmonary resection with major vascular reconstruction, or a consequence of trauma (Wilson et al. 1993; Williams et al. 1996; Jordan et al. 2000; King et al. 2000; Porhanov et al. 2002; Ng et al. 2002; Erdogan et al. 2005).

The pathophysiology of IR injury in the lung involves increased leakage from the pulmonary microvasculature leading to interstitial and alveolar edema, excessive infiltration of polymorphonuclear cells into the lung, tissue inflammation, and apoptosis (de Perrot et al. 2003).

The inflammatory response that underlies IR injury of the lung was demonstrated to be biphasic, with an acute macrophage-dependent phase followed by a later phase characterized by neutrophil recruitment and activation (Eppinger et al. 1995; Fiser et al. 2001). There is ample evidence supporting a prominent role for alveolar macrophages in the initiation and propagation of acute lung IR injury (Eppinger et al. 1997; Naidu et al. 2003). Pro-inflammatory cytokines and chemo kines, such as TNF-α and monocyte chemoattractant protein-1 (MCP-1), respectively, are secreted early by alveolar macrophages after reperfusion and are postulated to contribute to the pathogenesis of lung IR injury (Mayer et al. 1993; Krishnadasan et al. 2003; Maxey et al. 2004; Zhao et al. 2006). Moreover, an *in vitro* model of pulmonary IR has demonstrated that following reperfusion, alveolar macrophage-derived TNF-α further induces alveolar epithelial cells to produce key chemokines that may contribute to subsequent lung injury through the recruitment of neutrophils (Sharma et al. 2007). Together, these observations suggest that alveolar macrophages are activated early by IR and initiate a cascade of events leading to a robust inflammatory response.

Lung IR, like other organs undergoing IR, is characterized by the occurrence of apoptosis. In a model of lung transplantation in rats (Fischer et al. 2000b) and following lung transplantation in human (Fischer et al. 2000a), apoptosis of pneumocytes after reperfusion of the transplanted lung was found to be a very early event that peaks at 2 h (Borghi-Scoazec et al. 1997; Ikeda et al. 1998; Burns et al. 1998; Freude et al. 2000; Fischer et al. 2000a; Fischer et al. 2000b; Regan et al.

2003). Mitogen-activated protein kinases (MAPKs) are serine-threonine protein kinases that participate in the anti-inflammatory /inflammatory cell signaling of processes such as IR injury (Lai et al. 2004). In the lung, activation of the MAPK pathway has been postulated to be involved in mediating IR-induced pneumocyte apoptosis and lung dysfunction (Abe et al. 2000). Extracellular signal-regulated kinase (ERK), c-Jun amino-terminal protein kinases (JNK) and the p38 kinase (p38), all belonging to the MAPK family, were shown to be activated in lung IR injury, to different extents (Bogoyevitch et al. 1996; Abe et al. 2000; Toledo-Pereyra et al. 2004; Lai et al. 2004; Khan et al. 2004). In the current medical literature on management of IR syndromes, MAPKs are emerging as important targets for study. It has been noted that whereas ERK1/2 exerts a cytoprotective effect and is involved in cell proliferation, transformation, and differentiation, p38 and JNK promote cell injury and apoptosis (Xia et al. 1995; Yue et al. 2000; Chang and Karin 2001; Toledo-Pereyra et al. 2004; Khan et al. 2004).

11.3.1.2 The Role of A_3AR in the Attenuation of IR-Induced Lung Injury

Multiple studies have illustrated the protective role of adenosine in IR injury in various organs including the heart, kidney, liver, and lung (Cronstein et al. 1990, 1992). The role of A_3AR in attenuating IR lung injury has also been demonstrated. In an *in vivo* spontaneously breathing cat model, the selective activation of A_3AR with reperfusion (by either IB-MECA or the newly synthesized A_3AR analog, MRS3558) reduced IR lung injury and associated apoptosis (Table 11.2; Fig. 11.1)

Table 11.2 The effects of A_3 agonist (IB-MECA) and antagonist (MRS-1191) on IR-induced lung injury in spontaneously breathing cats

Group	Injured alveoli (%)	MPO activity[a]	Wet/dry weight ratio
I	2.7 ± 1.2[b]	1.3 ± 0.2[b]	4.9 ± 0.5[b]
II	49.2 ± 3.1	4.7 ± 0.5	8.1 ± 0.6
III	17.1 ± 2.5[b]	2.1 ± 0.3[b]	4.4 ± 0.4[b]
IV	18.7 ± 3.0[b]	1.9 ± 0.2[b]	4.6 ± 0.4[b]
V	23.1 ± 3.3[b]	2.3 ± 0.3[b]	4.6 ± 0.3[b]
VI	44.8 ± 3.3	4.9 ± 0.6	8.0 ± 0.5

The effects of A_3AR agonist and antagonist on IR-induced lung injury are represented via parameters of injury, i.e. percentage of injured alveoli, tissue myeloperoxidase (MPO) activity, and lung tissue wet:dry weight ratio. The groups were as follows: I, nonischemic group; II, ischemia-reperfusion; III, IB-MECA was administered before ischemia; IV, IB-MECA was administered 1 h after beginning of ischemia; V, IB-MECA was administered 5 min after beginning of reperfusion; VI, MRS-1191 pretreatment before IB-MECA, as in group III, and beginning of ischemia. Values shown are means ± SEM; n = 6 cats/group.

[a] Tissue myeloperoxidase (MPO) activity is expressed in units of myeloperoxidase/g of lung weight, each of which was defined as the activity degrading 1 μmol of peroxide per min at 25°C.

[b] $p < 0.05$ compared with group II and VI (obtained from Rivo et al. 2004).

Reperfusion, but not ischemia, caused significant injury. Intravenous administration of IB-MECA before or after ischemia or after reperfusion resulted in significant attenuation of lung injury.

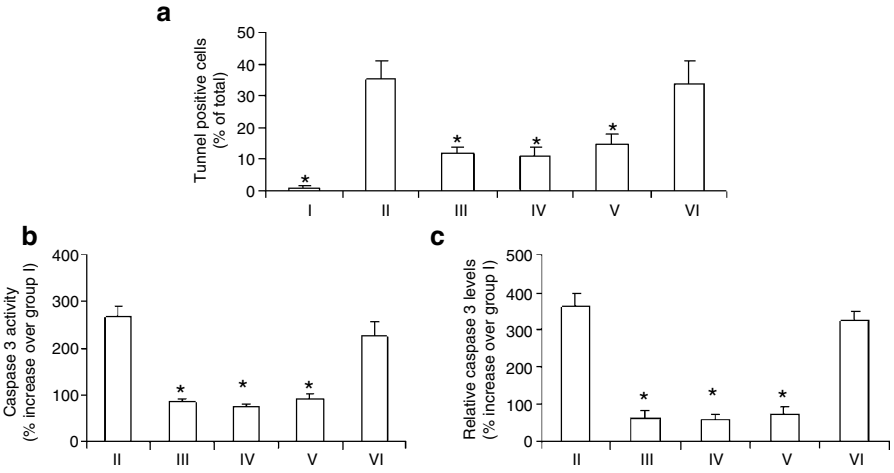

Fig. 11.1 The effects of A_3AR agonist (IB-MECA) and antagonist (MRS-1191) on pneumocyte apoptosis following *in vivo* reperfusion. Apoptosis was evaluated by Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells (**a**), changes in caspase 3 activity (**b**) and changes in activated caspase 3 expression as analyzed by Western blotting (**c**). Groups included in the study: (I) nonischemic group; (II) ischemia-reperfusion (IR); (III) pretreatment with IB-MECA (before ischemia); (IV) IB-MECA administration 1 h after beginning of ischemia; (V) IB-MECA administration 5 min after beginning of reperfusion; (VI) MRS-1191 pretreatment before IB-MECA, as in group (III) and beginning of ischemia. Values shown are means ± SEM. *$p < 0.05$ compared to groups (II) and (V). Continuous perfusion of the lung induced no apoptosis (group I). Reperfusion resulted in significant lung injury and apoptosis (group II). The administration of the A_3AR agonist before ischemia, after ischemia or with reperfusion attenuated lung injury and apoptosis (groups III–V). This protective effect was reversed in animals pretreated with the A_3AR antagonist (obtained from Rivo et al. 2004)

(Rivo et al. 2004a-b; Matot et al. 2006). Matot et al. have further utilized this *in vivo* animal model to evaluate whether A_3AR activation has a sustained effect beyond the first 3 h. Administration of a single dose of a selective A_3 agonist prior to reperfusion improved the immediate response to reperfusion, with reduced lung edema, inflammation and apoptosis. This protective effect was sustained at 27 h after reperfusion (Matot et al. 2008). The protective impact of A_3AR agonists was not accompanied by hemodynamic adverse effects: intravenous administration of IB-MECA did not produce any effect on heart rate or blood pressure, suggesting that at the dose used in that study (300 μg/kg), this agent does not interact with A_1 or A_{2A} receptors in cats. Nor did it have any effect on plasma histamine concentration, implying that agonist therapy does not involve degranulation of resident mast cells within the lung. Administration of a nitric oxide synthase inhibitor, in doses previously shown to block enzyme activity in this model (Cheng et al. 1996) did not abolish IB-MECA–induced lung protection, whereas, K_{ATP} channel–blockade (Cheng et al. 1996) significantly reduced the protective effect of IB-MECA. Taken together, these results demonstrate that A_3AR agonists confer powerful protection against IR-induced lung injury which involves K_{ATP} channel opening,

and that this protectivity in the lungs does not require nitric oxide synthase activity (Rivo et al. 2004).

11.3.1.3 Postulated Mechanisms of A_3 Adenosine Receptor-Mediated Lung Protection

Few potential mechanisms may explain the protective effects of adenosine, and of A_3AR in particular, during IR injury. The first involves its anti-inflammatory activity. As mentioned before, reperfusion injury has been reported to be mediated by a variety of cellular and humoral factors, including platelets, cytokines, cell adhesion molecules, and neutrophils, which migrate and release proinflammatory mediators and reactive oxygen species (Al-Mehdi and Fisher 1998). A_3AR has been linked to a variety of anti-inflammatory processes, including inhibition of lipopolysaccharide-induced secretion of TNF-α production from murine (Bowlin et al. 1997) and human (Sajjadi et al. 1996) macrophage-like cell lines, inhibition of PAF-induced chemotaxis of human eosinophils (Walker et al. 1997), inhibition of neutrophil-mediated tissue injury (Jordan et al. 1999) and inhibition of neutrophil degranulation (Bouma et al. 1997).

A second complementary mechanism by which A_3AR activation may attenuate IR lung injury is the inhibition of apoptosis. Of the four G protein-coupled ARs, the A_3 receptor has been specifically implicated in the modulation of cell death and survival. Results obtained by exposing both cardiomyocytes and astrocytes to graded concentrations of selective A_3AR agonists suggest induction of both cell protection and cell death, an opposite effect, which likely depends on the degree of receptor activation (Abbracchio et al. 1997; Shneyvays et al. 1998, 2000; Jacobson et al. 1999). Using an A_3-selective agonist, Rivo et al. have found that A_3AR plays an anti-apoptotic role during lung IR in-vivo (Rivo et al. 2004, Fig. 11.1). Different molecular mechanisms have been described in the literature to explain the anti-apoptotic effect of A_3AR. Reinforcement of cytoskeleton functions leading to a better adherence of cells and hence a reduced susceptibility of cells to apoptosis has been suggested by *in vitro* studies in astroglial cells and cardiomyocytes (Abbracchio et al. 1997; Shneyvays et al. 1998). In addition, modulation of Bcl-2-like protein by A_3AR has been noted, raising the possibility that A_3AR-mediated modulation of cell survival may occur through a specific action on the cytoskeleton, mediated by Bcl-2-like proteins (Jacobson et al. 1999). Also, treatment with A_3AR agonist was associated with sustained down-regulation of phospho-p38 levels – which otherwise increases with reperfusion – known to promote cell injury and apoptosis (Xia et al. 1995; Chang and Karin 2001). Previous studies reported that activation of the p38-related intracellular signal pathway induces TNF-α and IL-1β production, and vice versa (Lee et al. 1994). Accordingly, in a warm IR injury model in rat lung, Kawashima et al. revealed that the ischemic injury may cause activation of p38 with subsequent TNF-α and IL-1β production (Kawashima et al. 2001). A complex interaction exists between TNF-α, IL-1β, and p38 to promote reperfusion

lung injury. Concurrently with the inhibition of apoptotic pathways, A_3AR has been shown to promote cell survival. Attenuation of IR lung injury with A_3AR was associated with upregulation of phosphorylated ERK levels at 3 h of reperfusion (when compared to reperfusion without treatment), thus promoting cytoprotective pathways (Xia et al. 1995). Taken together, these data suggest that treatment with the A_3AR agonist causes changes in the MAPK pathway to shift the balance between cell death and survival in favor of cell survival.

Alternatively, A_3AR has been demonstrated to activate phosphatidylinositol 3-kinase (PI3K), and subsequently the serine/threonine protein kinase B (PKB/Akt), also known as the PI3K-PKB/Akt pathway (Chan et al. 1999; Gao et al. 2001). PKB/Akt acts as a cell survival factor by modulating the level of caspases, the expression of anti-apoptotic genes and the level of GSK-3β, a key protein in the Wnt signaling pathway (Krasilnikov 2000; Fishman et al. 2002), thus prolonging cell survival and maintaining the accumulation of those cells in inflamed tissues. The PI3K-PKB/Akt pathway may be up or down-regulated upon A_3AR activation, in a cell type-specific manner.

11.3.1.4 Other Adenosine Receptor Subtypes Involved in Lung Reperfusion Injury

Targeting other AR subtypes has also proved beneficial in the treatment of IR-related lung injury. In the rabbit and rat lungs, as well as in intact-chest, spontaneously breathing cats, the nonselective adenosine analog 2-chloroadenosine as well as an A_{2A}AR agonist significantly decreased the severity of IR injury (Marts et al. 1993; Khimenko et al. 1995; Fiser et al. 2002; Rivo et al. 2007). A_{2A}AR activation was associated with decreased apoptosis, and involved ERK1/2 activation and alterations in the expression of antiapoptotic Bcl-2 and proapoptotic Bax proteins (Rivo et al. 2007). Similarly, in an *in vivo* model of porcine lung transplantation, activation of A_{2A}AR reduced inflammation and preserved pulmonary function (Reece et al. 2005). A_1AR blockade and A_{2B}AR activation were also efficient attenuators of IR lung injury in a feline and murine model, respectively (Neely and Keith 1995; Eckle et al. 2008). The later acted by enhancing alveolar fluid clearance in a mouse model of ventilator-induced lung injury (Eckle et al. 2008). Taken together these data indicate that treating IR lung injury via the adenosine pathway may be approached by manipulating either of the various adenosine receptor subtypes.

11.3.2 Adenosine A_3 Receptor in the Setting of Other Etiologies of Lung Injury

Polymorphonuclear neutrophils (PMN) have been implicated in the pathogenesis of post-traumatic and sepsis-related complications such as acute lung injury and acute respiratory distress syndrome (ARDS), a prominent form of end-organ damage that

is frequently observed in patients even in the absence of direct mechanical trauma to the lungs (Ware and Matthay 2000; Durham et al. 2003). PMNs infiltrate lung tissues, occlude pulmonary capillaries, release cytotoxic mediators such as neutrophil proteases, and consequently destroy lung tissues (Ware and Matthay 2000; Abraham 2003; Inoue et al. 2005, 2006). Inoue et al. have demonstrated that A_3AR and the purinergic receptor P2Y2 are involved in neutrophil sequestration to the lungs in a mouse model of sepsis (Inoue et al. 2008a). In that study $A_3^{-/-}$ mice exhibited reduced pulmonary damage, and PMN migration into the lung was decreased. Leukocytes of these mice also failed to accumulate adequately in the intra-abdominal compartment, but survival of $A_3^{-/-}$ mice was prolonged. The authors attributed prolonged survival to the reduced secondary lung damage that had been observed in $A_3^{-/-}$ mice (Inoue et al. 2008). Thus, pharmaceutical approaches that target these receptors might be useful to control acute lung tissue injury in the setting of sepsis.

Previous studies have shown that murine PMN express predominantly the A_{2A}AR and A_3AR subtypes, and that A_{2A}AR inhibits formyl methionylleucyl-phenylalanine (fMLP)-stimulated PMN degranulation, whereas A_3AR augments this response (Chen et al. 2006). Various animal models as well as *in vitro* experiments with isolated human PMN have shown that hypertonic saline resuscitation can significantly reduce the risk of post-traumatic complications by inhibiting excessive PMN activation (Junger et al. 1994; Angle et al. 1998; Murao et al. 2000, 2003). However, timing of the hypertonic saline treatment was found to be crucial for the prevention of lung damage: whereas early treatment with hypertonic saline inhibits PMN activation and thus prevents lung injury, delayed hypertonic saline resuscitation aggravates lung tissue damage (Murao et al. 2000; Hashiguchi et al. 2007). Using a mouse model of sepsis via cecal ligation and puncture, Inoue et al. have shown that A_3AR expression on PMN determines whether hypertonic saline resuscitation inhibits or aggravates PMN-induced acute lung injury (Inoue et al. 2008). These findings suggest that A_3AR antagonists could improve the efficacy of hypertonic saline resuscitation by reducing side effects in patients whose PMNs are activated before hypertonic saline treatment.

Acute lung injury may also develop secondary to another clinical pathology, such as intestinal ischemia–reperfusion injury. Regardless of whether intestinal IR injury is the patients' primary pathophysiologic issue, or is secondary to other disease states, progression to the systemic inflammatory response syndrome (SIRS) often ensues (Yasuhara 2005; Stallion et al. 2005). Development of ARDS and acute renal failure (ARF) are two common sequelae of SIRS in critically ill patients (Rangel-Frausto et al. 1995). Since purinoceptors have been implicated in many of the pathophysiologic phenomena associated with the progression of SIRS to multiple organ failure (Di Virgilio et al. 2001; McCallion et al. 2004; Bulanova et al. 2005; Douillet et al. 2006), Milano et al. sought to examine both lung and kidney tissues for purinergic receptor expression changes following intestinal IR injury, in order to determine if extracellular nucleotides and their receptors are involved in the extra-intestinal manifestations of this disease process. They found that the expression of the purinoceptors A_3AR and P2Y2 is elevated in the murine lung and kidney, but not intestine following experimental intestinal IR injury (Milano et al. 2007)

suggesting a role for these receptors in remote organ injury. At this time, it is unclear whether elevation of A_3AR expression in the lung following intestinal IR injury has a protective or deleterious effect, and the clinical significance remains to be determined.

Acute lung injury may result from injurious mechanical ventilation as well. Large-volume mechanical ventilation can produce histologic lung injury, pulmonary edema, capillary stress failure, local and systemic inflammation, and, ultimately, increased mortality (Slutsky 1999). Mechanical ventilation was found to alter the extracellular adenyl-nucleotide profile and purinoceptor expression in the lung and extra-pulmonary tissues of rats. However, it mainly affected the levels of A_{2B}AR mRNA in the lung, whereas A_3AR message was unresponsive (Douillet et al. 2005).

Evidence for exacerbating effects of A_3AR activation on lung injury was exhibited in rodents (Guo et al. 2001; Young et al. 2004; Schepp and Reutershan 2008). Pulmonary inflammation was attenuated by pharmacological blockade or genetic removal of A_3AR, suggesting its proinflammatory effects. Accordingly, eosinophil levels and, although less pronounced, all other leukocytes – including PMNs – were reduced in the BAL fluid after treatment with an A_3AR antagonist (Young et al. 2004).

In summary, in the setting of lung injury, most animal studies point to a protective effect induced by A_3AR activation. Data on A_3AR function in human pulmonary disease are not available. However, degranulation of human PMNs has been demonstrated to be under the control of A_3AR (Bouma et al. 1997; Jordan et al. 1999), which suggests that A_3AR might well be involved in pulmonary inflammation.

11.4 Adenosine A_3 Receptor and Its Role in Modulation of Systemic and Pulmonary Vascular Tone

Adenosine affects vascular smooth muscle tone in the pulmonary circulation. In the feline pulmonary vascular bed, under conditions of controlled pulmonary blood flow and constant left atrial pressure, adenosine was shown to produce dose-dependent, tone-dependent responses (Neely and Matot 1996; Cheng et al. 1996). At low baseline pulmonary vascular tone adenosine induces vasoconstriction via A_1AR and the release of prostanoids, whereas at elevated pulmonary vascular tone it produces vasodilatation by acting on A_2AR, without nitric oxide release or the activation of guanylate cyclase or K_{ATP} channels (Neely and Matot 1996; Cheng et al. 1996).

In human as well as in some experimental models, such as the canine (Mentzer et al. 1975) and feline (Neely et al. 1989), adenosine has been demonstrated to act as a vasodilator in the lungs during conditions of elevated tone. However, the contribution of A_3AR to this effect is not clear. Earlier studies have postulated A_2 receptors to mediate adenosine-induced vasodilatation (Berne 1963; Phillis et al. 1987; McCormack et al. 1989). In the pulmonary circulation of rabbits and rats, adenosine has been reported to produce vasodilation via A_{2A}AR or A_{2B}AR, respectively (El-Kashef et al. 1999; Haynes et al. 1995, 1999). Accordingly, in an isolated blood-perfused rat lung, adenosine-induced vasodilatation was shown to be

mediated by A_{2B} receptors, through a nitric oxide-independent mechanism (Haynes et al. 1995). Adenosine was also shown to relax isolated human pulmonary arterial rings via an A_2AR-mediated mechanism (McCormack et al. 1989).

In a study performed on cardiac surgical patients, adenosine was demonstrated to act as a selective pulmonary vasodilator, without affecting systemic blood pressure (Fullerton et al. 1996). In this work, ten patients were studied in the operating room under general anesthesia, following cardiopulmonary bypass. Pulmonary vascular resistance (PVR), systemic vascular resistance (SVR), mean pulmonary arterial pressure (MPAP), and mean systemic arterial pressure (MAP) were determined before, during, and after central venous infusion of adenosine (50 pg/kg/min) for 15 min. Under these conditions, adenosine produced a significant reduction in MPAP, transpulmonary gradient, and PVR without changes in mean MAP or SVR. As adenosine is cleared from the blood by erythrocytes and vascular endothelial cells of the pulmonary circulation, its effect on the systemic vasculature when administered centrally is limited. The authors therefore suggested that central venous infusion of adenosine may be used therapeutically to relax pulmonary vascular smooth muscle.

Systemically, in addition to a direct A_2AR–mediated vasodilatation, adenosine causes vasoconstriction of isolated perfused arterioles from the hamster cheek pouch, which is often masked by vasodilatation (Doyle et al. 1994; Shepherd et al. 1996). This response to adenosine and its metabolite, inosine, was found to be due to stimulation of perivascular mast cells and the subsequent release of vasoconstrictors.

Further *in vivo* studies revealed that the arteriolar constriction that follows mast cell activation via inosine, is the result of histamine and thromboxane release and that A_3AR is involved in mediating this response (Shepherd and Duling 1996; Shepherd et al. 1996; Fozard et al. 1996; Reeves et al. 1997). Inosine, which does not bind to A_1 or A_2 receptors, thus elicits a monophasic arteriolar constrictor response distinct from the multiphasic dilator/constrictor response to adenosine (Jin et al. 1997).

Conversely, a correlation between A_3AR activation and hypotension through non-immunologically dependent mast cell mediator release has recently been demonstrated *in vivo*. The hypotensive response to A_3AR activation by N^6-2-(4-aminophenyl)-ethyladenosine (APNEA), a non-selective A_3AR agonist (Carruthers and Fozard 1993a, b; Collis and Hourani 1993), in the anesthetized rat was qualitatively similar to compound 48/80, a potent mast cell degranulator (Gietzen et al. 1983; Gietzen 1983). Additionally, the hypotensive response to APNEA was suppressed by mast cell degranulation inhibitors, and depletion of mast cell mediators with compound 48/80 decreased the effectiveness of APNEA-induced hypotension (Hannon et al. 1995). Van Schaick et al. (1996) have shown that intravenous administration of the A_3AR agonist, 2-Cl-IB-MECA, in conscious rats resulted in a hypotensive response coincident with an increase in plasma histamine concentrations indicating the involvement of the A_3AR in mediator release from mast cells *in vivo*. Therefore, A_3AR-mediated mast cell degranulation has been demonstrated to induce both vasoconstrictor and vasodilator responses in animal models.

A_3AR agonists IB-MECA and Cl-IB-MECA were shown to exert species-dependent hemodynamic effects in the pulmonary and cardiovascular system (Lasley et al. 1999).

In the pig both agonists increased pulmonary artery pressure, whereas in the rabbit heart neither agent exerted effects, and in the rat heart both agonists produced coronary vasodilation. In addition, this dilatory effect was minimally altered by the A_3AR antagonist MRS-1191, but was completely blocked by the adenosine A_{2A}AR antagonist Sch-58261. Therefore, vasodilation of the coronary vessels appears to be mediated primarily by A_{2A}AR activation (Lasley et al. 1999). In the intact pig IB-MECA and Cl-IB-MECA both produced a profound, but reversible, systemic hypotension and pulmonary hypertension. The results of this study indicated that although these agents have been reported to be protective in the ischemic-reperfused rabbit heart (Tracey et al. 1997; Hill et al. 1998), further studies on the exact role of cardiovascular A_3ARs need to be performed in additional species and intact animal preparations (Lasley et al. 1999).

Finally, Rivo et al. (2004) and others (Neely and Keith 1995; Jiang et al. 1998; Matot and Jurim 2001) have demonstrated that exposure of the lung to ischemia and reperfusion causes a prompt increase in pulmonary artery pressure followed by a gradual decline. However, compared with the baseline values, the pressure remains elevated during the remainder of lung reperfusion. The increase in lobar arterial pressure observed during the reperfusion period was significantly smaller in the groups administered with A_3AR agonist before ischemia or reperfusion. However it is yet to be elucidated whether this is a direct effect on vascular A_3ARs, or a consequence of a milder inflammation of the lung.

Taken together, the data presented here indicate that in general adenosine affects pulmonary blood pressure in a species-, receptor-, dose- and tone-dependent manner. Adenosine is known as an endogenous vasodilator, and its role in the coronary and other circulations is well recognized. Exogenous adenosine may serve as a short acting vasodilator. In certain conditions, however, it may excert vasoconstriction as well. The biological functions of A_3AR in the pulmonary circulation are ambiguous. A_3AR was shown to mediate both vasoconstrictor and vasodilator responses in the lungs of animal models, in a species-dependent manner. In man, the relevance of A_3AR to adenosine-induced effects on the pulmonary vasculator is yet to be investigated.

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Chapter 12
A_3 Adenosine Receptor Regulation of Cells of the Immune System and Modulation of Inflammation

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12.1 A_3 Adenosine Receptor Effects on Neutrophil Function

Neutrophils represent a larger percentage of circulating leukocytes than any other cell type. They are the first white blood cells to arrive at an injured or infected site. Neutrophils arise in the bone marrow and then must traverse the vasculature to arrive at the sites of injury. They leave the circulation at the level of the postcapillary venules following specific interaction with endothelium. Once in the extravascular space, neutrophils follow a gradient set up by chemoattractants, such as activated complement components, cytokines, lipids or bacterial products by means of specific cell surface receptors. Although the primary role of the neutrophil is to rid the body of injurious organisms and clean up the debris after tissue injury, the extracellular release of any of the contents of the phagolysosome or the generation of toxic oxygen metabolites into the extracellular space can lead to destruction of normal, uninjured cells surrounding the infected site. It is the destruction of the surrounding tissue by overactive neutrophils that adds so greatly to tissue destruction in the setting of reperfusion injury. Adenosine, acting through its cell surface receptors, is a potent regulator of neutrophil function.

The first report implicating a role for A_3 receptors in human neutrophils came in 1997 following investigations into the effect of adenosine and its more selective analogues on neutrophil degranulation in human whole blood (Bouma et al. 1997). Adenosine inhibited concentration-dependently the LPS- and TNF-alpha-induced release of the azurophilic granule proteins with an IC_{50} in the μmolar range. The inhibitory effects of adenosine were partially blocked by the A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine, the A_1/A_2 antagonist 8(p-sulfophenyl) theophyline, and the A_1/A_3 antagonist xanthine amine congener, but not by the

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A_1 antagonist 1,3-dipropyl-8-cyclopentylxanthine. The highly selective A_3 agonist N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide and the nonselective agonist 2-chloroadenosine reduced degranulation more potently than the A_1 agonist N6-cyclopentyladenosine. The inhibitory effects of N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide and 2-chloroadenosine were strongly reversed by xanthine amine congener, but were not affected by 8(p-sulfophenyl)theophyline. These data suggest that adenosine acted via A_2 as well as A_3 receptors to inhibit neutrophil degranulation. However, activation of A_3 receptors in canine neutrophils did not attenuate superoxide anion production but reduced platelet-activating factor-stimulated neutrophil adherence to coronary endothelium suggesting that it might be a novel target for treatment of myocardial ischemia and reperfusion (Jordan et al. 1999).

Subsequent binding and functional studies showed that human neutrophils expressed A_3 receptors which were coupled to the inhibition of adenylyl cyclase and calcium signalling (Gessi et al. 2002). However in the case of calcium the high micromolar doses of the A_3 agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-*N*-methylcarboxamide (Cl-IB-MECA) and the A_3 antagonist 5-*N*-(4-methoxyphenylcarbamoyl)amino-8-propyl-2(2furyl)-pyrazolo-[4,3e]-1,2,4-triazolo [1,5-c] pyrimidine (MRE 3008F20) needed to stimulate or block Ca^{2+} mobilization respectively, were not completely consistent with the involvement of an A_3 receptor. Similar effects of Cl-IB-MECA in mobilizing Ca^{2+} have been found in several cell systems a finding that is difficult to reconcile with the high affinity of this selective A_3 agonist in binding and cAMP inhibition assays (Kohno et al. 1996a, b; Jacobson 1998; Reeves et al. 2000; Reshkin et al. 2000; Shneyvays et al. 2000; Gessi et al. 2001; Suh et al. 2001; Merighi et al. 2001). The reason why high, nonselective doses of Cl-IB-MECA are needed to stimulate Ca^{2+} mobilization remains unknown. A contribution of other mechanisms other than A_3 receptor stimulation cannot be excluded. Importantly, for the first time it was suggested that both A_3 and A_{2A} receptors contribute to the inhibition of oxidative burst, an indication of anti-inflammatory activity (Gessi et al. 2002). Using this readout, alterations of A_3 adenosine receptors in human neutrophils exposed to low frequency, low energy pulsing electromagnetic fields (PEMFs) has been reported. There is considerable interest in the use of PEMFs in clinical practice since the date correlate well with inflammatory conditions. Saturation experiments after treatment with PEMFs revealed that the A_3 receptor density in human neutrophils was increased. Consistent with this in functional assays Cl-IB-MECA and N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (IB-MECA) were able to inhibit cyclic AMP accumulation and their potencies were increased after exposure to PEMFs. These results indicated that in human neutrophils treated with PEMFs there are significant alterations in the A_3 adenosine receptor density and functionality (Varani et al. 2003). The upregulation cannot be ascribed to the synthesis of new receptors since the duration of PEMF treatment was too short. The upregulation of A_3 adenosine receptors is most likely due to a translocation of this receptor subtype to the membrane surface. It is of interest that PEMFs treatment also modified the binding parameters of the A_{2A} adenosine receptors but not those of α_2, β_2 adrenergic and μ, k opioid receptors suggesting a

relationship between adenosine receptor-mediated anti-inflammatory effects and PEMF exposure (Varani et al. 2002).

An up-regulation of the A_3 adenosine receptor has also been observed in neutrophils obtained from patients with colorectal cancer in comparison with healthy subjects. This overexpression was found to reflect at peripheral level the same up-regulation found in the tumoral tissue from the colon in comparison to healthy mucosa, suggesting that peripheral A_3 adenosine receptors in neutrophils might represent potential marker for revealing colorectal cancer (Gessi et al. 2004a). It was also found that in a small cohort of subjects A_3 receptor expression of circulating blood cells normalizes after surgical treatment, consistent with the negative results of follow-up evaluation with carcinoembryonic antigen (CEA), computed tomography scan, and colonoscopy. Hence, the improved health of patients after surgical resection seems to be associated with restoration of a normal adenosinergic system, at least in terms of A_3 receptor expression. These findings might be used for clinical applications. In particular, examination of neutrophil A_3 expression (e.g., in addition to CEA determination) could play a role in the screening of high-risk individuals or in the follow-up of patients after surgical resection.

Recently Chen et al. (2006a) reported that migrating human neutrophils secrete ATP at the leading edge, which signals via P2Y2 receptors to amplify chemoattractant signals. Neutrophils rapidly hydrolyze released ATP to adenosine which then acts via A_3 receptors, which are recruited to the leading edge, to promote cell migration. In resting cells, A_3 receptors appear to be located primarily in intracellular compartments associated with granules (Chen et al. 2006a). Upon cell stimulation with chemoattractant, A_3 receptors are rapidly mobilized at the leading edge to promote chemotaxis. Thus, ATP release and autocrine feedback through P2Y2 and A_3 receptors provides signal amplification and controls gradient sensing and migration of neutrophils. Interestingly, chemotaxis of neutrophils obtained from A_3 receptor knockout (KO) animals is inhibited. In contrast, A_{2A} receptors are uniformly distributed across the cell surface and cell polarization does not seem to change this distribution pattern (Chen et al. 2006a). This suggests that the inhibitory A_{2A} receptors may function to globally suppress pseudopod formation across the entire cell surface of neutrophils, except at the leading edge, where A_3 adenosine receptor counteract the suppressive action of A_{2A} receptors (Chen et al. 2006a; Linden 2006; Junger 2008). However, these findings were questioned recently by van der Hoeven et al. (2008) who demonstrated that A_3 adenosine receptor activation is responsible for inhibition of superoxide production and chemotaxis of mouse bone marrow neutrophils, suggesting that the A_3 receptor may contribute to the anti-inflammatory actions of adenosine. Although there are many differences between this study and the earlier work, including the species difference (mouse vs human), the pharmacological agents used to stimulate the A_3 adenosine receptor (CP-532,903 versus IB-MECA), the methods used to isolate/culture murine neutrophils, the stimulation protocols (including the time and duration of pretreatment of cells with agonists), and the state of cell priming, a definite explanation for the differences in results obtained in these studies remains unclear.

Consistent with a pro-inflammatory role of A_3 adenosine receptors in human neutrophils it has been demonstrated that A_3 receptors together with P2Y subtypes mediate neutrophil elastase release induced by hypertonic saline (Chen et al. 2006b). Hypertonic saline holds promise as a novel resuscitation fluid for the treatment of trauma patients because it inhibits polymorphonuclear neutrophil activation and thereby prevents host tissue damage and associated post-trauma complications. However, under certain conditions of cell activation, hypertonic saline can increase neutrophil degranulation, which could exacerbate tissue damage in trauma victim (Chen et al. 2006b). The cellular mechanism by which hypertonic saline increases degranulation involves elastase release and ERK and p38 MAPK activation when hypertonic saline is added after submaximal activation of neutrophils with formyl peptide (fMLP) or phorbol ester (PMA). Agonists of P2 nucleotide and A_3 adenosine receptors mimicked these enhancing effects of hypertonic saline, whereas antagonists of A_3 receptors or removal of extracellular ATP with apyrase diminished the response to hypertonic saline suggesting that hypertonic saline upregulates degranulation via ATP release and positive feedback through P2 and A_3 receptors. It has been hypothesized that these feedback mechanisms can serve as potential pharmacological targets to fine-tune the clinical effectiveness of hypertonic saline resuscitation (Chen et al. 2006b). In this context, it has been shown that A_3 receptor activation may diminish the efficacy of hypertonic saline in a mouse model of acute lung injury after sepsis (Inoue et al. 2008a). Acute lung injury in wild-type mice treated with hypertonic saline 60 min after sepsis induction, through cecal ligation and puncture (CLP), was significantly greater than in wild-type mice pretreated for 5 and 15 min with hypertonic saline. Parallel experiments aimed at evaluating the expression of A_3 receptors in human neutrophils treated with hypertonic saline either 10 min before or after stimulation with formyl methionylleucyl-phenylalanine (fMLP) reveal that in the first condition A_3 receptor expression was reduced whilst in the second one it was markedly increased. These findings show that the opposing effects of hypertonic saline in vivo correlate with differences in the cell surface expression of A_3 receptors, suggesting that the enhancing effects of hypertonic saline are a result of increased A_3 receptor expression of stimulated neutrophils. The aggravating effect of delayed hypertonic saline treatment was absent in A_3 receptor knockout (KO) mice. Similarly, mortality in wild-type mice with delayed hypertonic saline treatment was significantly higher than in animals treated with hypertonic saline before CLP. Mortality in A_3 receptor KO mice remained at only 50% regardless of timing of hypertonic saline administration. These findings suggest that A_3 antagonists could improve the efficacy of hypertonic saline resuscitation by reducing side effects in patients whose polymorphonuclear neutrophils are activated before hypertonic saline treatment. The role of A_3 and P2Y2 receptors in neutrophil sequestration in the lungs in a mouse model of sepsis has also been demonstrated (Inoue et al. 2008b). Sepsis was induced by CLP using wild type mice, homozygous A_3 receptor KO mice, and P2Y2 receptor KO mice. The data suggest that A_3 and P2Y2 receptors are involved in the influx of neutrophils into the lungs after sepsis. Neutrophil sequestration in the lungs reached a maximum 2 h after CLP and remained significantly higher in wild type mice compared with A_3 KO and P2Y2 KO mice. Survival

after 24 h was significantly lower in WT mice than in A_3 KO or P2Y2 KO mice. Thus, pharmaceutical approaches that target these receptors might be useful to control acute lung tissue injury in sepsis.

It has been recognized that the inflammatory response to infection depends on the coordinated interaction of the adenine nucleotides, ATP, ADP and adenosine released by damaged tissue (Linden 2006). Therefore the contribution of A_3 receptors expressed in neutrophils, must be in concert with the other purinergic receptors to allow neutrophil adhesion, extravasation and chemotaxis. Neutrophils express predominantly A_{2A} and A_3 receptors which have opposite effects on these cells. In this chapter we have described how neutrophils following gradients of ATP and adenosine initiate and increase the speed of chemotaxis via P2Y and A_3 receptors, respectively and that the A_{2A} may amplify gradient signals by inhibiting chemotaxis at membrane region distant from the leading edge where the A_3 receptor predominates and increases chemotaxis. This seems possible given that the affinity of adenosine for the A_{2A} receptor is several orders of magnitude higher than its affinity for the A_3 receptors. Thus, differences in external adenosine concentrations in the environment surrounding migrating neutrophils may contribute to the regulation of chemotaxis (Chen et al. 2006a). A coordinated activity of A_{2A} and A_3 receptors has also been found with respect to degranulation and superoxide anion production in human neutrophils where both receptors cooperate to fine-tune the inflammatory response (Bouma et al. 1997; Gessi et al. 2002). However, it is important that the inhibitory effect exerted by A_{2A} and A_{2B} receptors on chemotaxis and adhesion to endothelial cells, respectively can overcome the stimulatory effect exerted by A_3 when excessive influx of neutrophils damages host tissues (Zhang et al. 2006). After activation of A_3 receptors opposite effects on inflammation have been reported depending essentially on the response considered, the experimental conditions and the species used (Gessi et al. 2008). It is relevant to underline that by comparing the studies performed in human neutrophils both anti and proinflammatory effects have been demonstrated (Fig. 12.1). Therefore caution should be used before proposing A_3 agonists as anti or proinflammatory agents until a more definite role of this receptor has been defined.

12.2 A_3 Adenosine Receptor Effects on Eosinophil Function

Eosinophils are one of the immune system components responsible for combating infection. Along with mast cells, they also control mechanisms associated with allergy and asthma. Eosinophils develop and mature in the bone marrow. They differentiate from myeloid precursor cells in response to the cytokines interleukin 3 (IL-3), interleukin 5 (IL-5), and granulocyte macrophage colony-stimulating factor (GM-CSF). Eosinophils produce and store many secondary granule proteins prior to their exit from the bone marrow. After maturation, eosinophils circulate in blood and migrate to inflammatory sites in tissues, in response to chemokines such as CCL11 (eotaxin-1), CCL24 (eotaxin-2), CCL5 (RANTES), and leukotriene B4

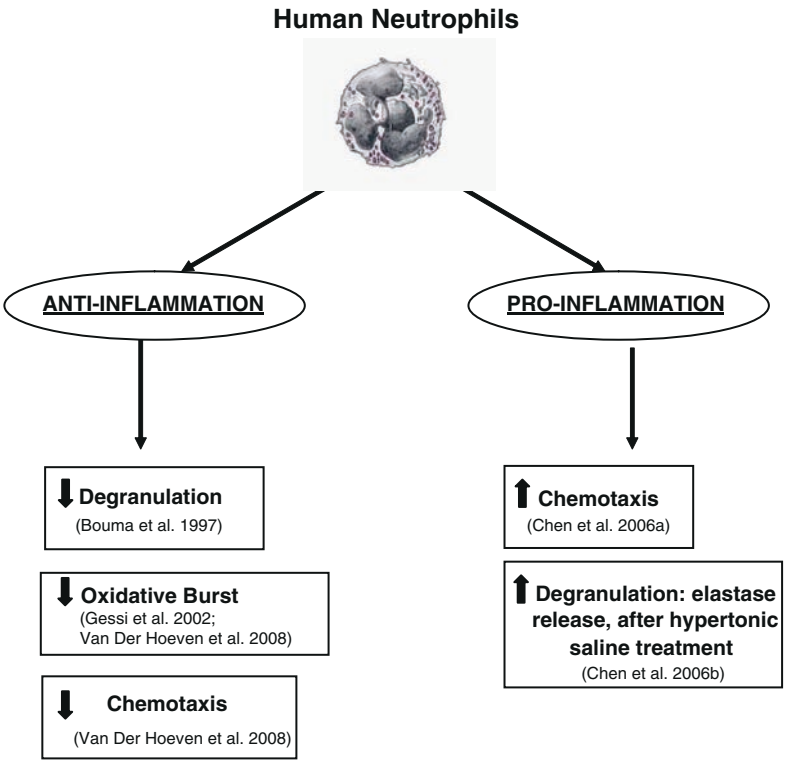

Fig. 12.1 Effects of A_3 adenosine receptors in neutrophils

(LTB4). At these infectious sites, eosinophils are activated by Type 2 cytokines released from a specific subset of helper T cells (T_h2); thus IL-5, GM-CSF, and IL-3 are important for eosinophil activation as well as maturation. Following activation, eosinophils release the contents of small granules within the cellular cytoplasm, which contain many chemical mediators, such as histamine and proteins such as eosinophil peroxidase, RNase, DNases, lipase, plasminogen, and major basic protein that are toxic to both parasite and host tissues (Gleich and Adolphson 1986).

A_3 receptors are present on human eosinophils and couple to signalling pathways that lead to cell activation (Kohno et al. 1996a; Reeves et al. 2000). Despite this it has not proven easy to demonstrate the functional consequences of activation of these sites (Reeves et al. 2000). Nevertheless, the chronic inflammation in asthma is characterised by extensive infiltration of the airways by activated eosinophils (Holgate 1999; Pearlman 1999) and it remains possible that the elevated adenosine concentrations associated with asthma would contribute to eosinophil activation through stimulation of A_3 receptors. In addition, it has been speculated that activation of A_3 receptors may protect eosinophils from apoptosis (Gao et al. 2001). Thus, blockade of A_3 receptors may reduce the numbers of eosinophils and their activation thereby reducing the pro-inflammatory burden in the lung. Consistent with this, following 6 weeks treatment of mild asthmatics with theophylline there was a

significant reduction in the number of activated eosinophils beneath the epithelial basement membrane (Sullivan et al. 1994). Significantly, the average blood levels in this study (37 μM) were within the range of the affinity of theophylline for the human A_3 receptor. Moreover, it has been reported that activation of A_3 receptors mediates inhibition of eosinophil chemotaxis (Knight et al. 1997). The authors argue that since adenosine levels are highest at the site of inflammation, A_3 receptor activation would be pro-inflammatory by inhibiting eosinophil migration away from the sites of inflammation. Clearly, however, inhibition of chemotaxis could be pro- or anti-inflammatory. In line with a pro-inflammatory role, a high expression of A_3 receptor transcripts has been found in eosinophilic infiltrates of the lungs of patients with asthma and chronic obstructive pulmonary disease (COPD) (Walker et al. 1997). Interestingly, similar findings were seen in the lungs of adenosine deaminase deficient ($ADA^{-/-}$) mice that showed adenosine-mediated lung disease. Treatment of $ADA^{-/-}$ mice with MRS 1523, a selective A_3 receptor antagonist, prevented airway eosinophilia and mucus production. Similar results were obtained in the lungs of ADA/A_3 receptor double KO mice, suggesting that A_3 receptor signalling plays an important role in regulating chronic lung disease and that A_3 receptor antagonism may be useful for reducing eosinophilia (Young et al. 2004). However these results contrast with those from experiments performed in human eosinophils ex vivo, where chemotaxis, degranulation and superoxide anion production were reduced by A_3 receptor activation (Knight et al. 1997; Walker et al. 1997; Ezeamuzie and Philips 1999). This discrepancy was later attributed to the *ex vivo* nature of the chemotaxis experiments and implied that diminished airway eosinophilia seen in the lungs of $ADA^{-/-}$ mice following disruption of A_3 receptor is not a direct effect on the eosinophils but be due to the modulation of key regulatory molecules from other cells that express A_3 receptors and that affect eosinophil migration (Young et al. 2004). For example A_3 receptors are expressed on murine mast cells, airway macrophages and epithelial cells, all of which might affect eosinophil migration. However levels of key regulatory cytokines such as IL-5 and IL-13, or chemokines including eotaxin I, thymus- and activation-regulated chemokine (TARC) and monocyte chemotactic protein-3 (MCP3) were not affected by A_3 receptor deletion in $ADA^{-/-}$ mice, pointing perhaps to the involvement of A_3 receptor in the regulation of other key modulators of eosinophil migration such as cell adhesion molecules, extracellular matrix elements and proteases (Young et al. 2004). In contrast to a pro-inflammatory role of the A_3 subtype implied by the work of Young and colleagues cited above, the involvement of the A_3 adenosine receptor in a bleomycin model of pulmonary inflammation and fibrosis seems to indicate an anti-inflammatory effect (Morschl et al. 2008). Analysis of A_3 adenosine receptor KO mice revealed enhanced pulmonary inflammation including an increase in eosinophils and a selective up-regulation of eosinophil related chemokines and cytokines in the lungs of A_3 adenosine receptor KO mice exposed to bleomycin. This increase in eosinophil numbers was accompanied by a decrease in the eosinophil peroxidase activity in lavage fluid from A_3 adenosine receptor KO mice exposed to bleomycin, an observation suggesting the A_3 adenosine receptor is necessary for eosinophil degranulation in this model. Together these results suggest that the A_3 adenosine receptor mediates anti-inflammatory functions in the bleomycin model, and is also involved in regulating

the production of mediators that can impact fibrosis (Morschl et al. 2008). The effects obtained in human eosinophils after A_3 receptor activation including inhibition of chemotaxis, degranulation, oxidative burst and the effects obtained from in vivo models of lung disease such as eosinophilia and mucus production are summarized in Fig. 12.2.

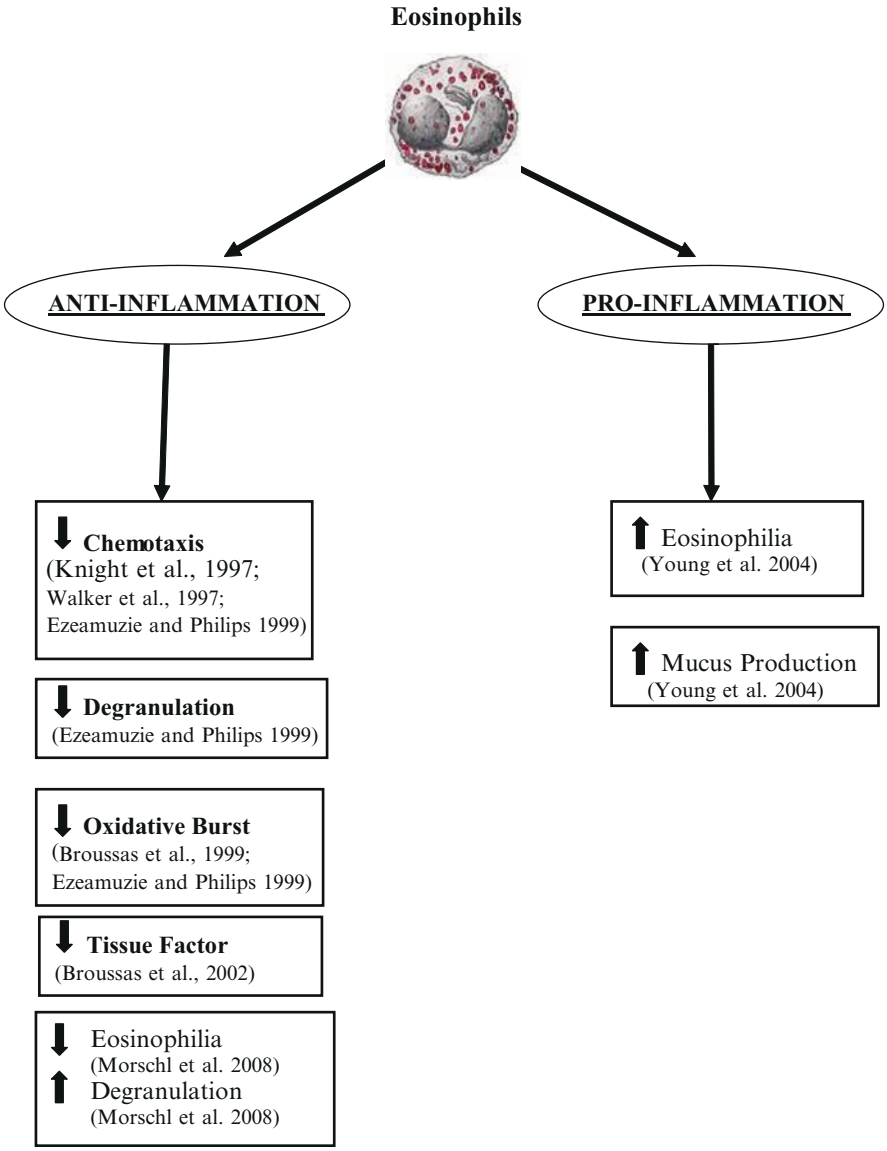

Fig. 12.2 Effects of A_3 adenosine receptors in eosinophils. Given the important species differences in the location and functional response to A_3 receptors, only the effects obtained in human are in *bold*

12.3 A_3 Adenosine Receptor Effects on Lymphocyte Function

The ability of immune cells to fight tumor cells is fundamental for successful host defence against cancer. Adenosine, whose concentration increases within hypoxic regions of solid tumors, may interfere with the recognition of tumor cells by cytolytic effector cells of the immune system (Blay et al. 1997; Merighi et al. 2003). Adoptive immunotherapy with lymphokine-activated killer (LAK) cells has shown some promise in the treatment of certain cancers that are unresponsive to conventional treatment approaches. However, colon adenocarcinomas tend to respond poorly to LAK therapy, possibly as a result of tumor-induced immunosuppression. It has been demonstrated that colon adenocarcinoma cells inhibited anti-CD3-activated killer cell induction through the production of a tumor-associated soluble factor that was distinct from transforming growth factor beta or prostaglandins (Hoskin et al. 1994a). As a result, adenosine was suggested as a possible inhibitor of killer T-cell activation in the microenvironment of solid tumours (Hoskin et al. 1994b, c). Indeed, evaluating the adhesion of murine spleen-derived anti-CD3-activated killer (AK) lymphocytes to syngeneic MCA-38 colon adenocarcinoma cells it was found that adenosine reduced adhesion by up to 60% (MacKenzie et al. 1994). The inhibitory effect of adenosine was exerted on AK cells and not on the MCA-38 targets and the agonist potency profile indicated that the A_3 receptor subtype might be responsible for the inhibition of adhesion. The authors suggested that this mechanism of immunosuppression, secondary to tissue hypoxia, may be important in the resistance of colorectal and other solid cancers to immunotherapy. In addition the same authors demonstrated that adenosine plays a strong inhibitory effect on the induction of mouse cytotoxic T cells (Hoskin et al. 2002). Diminished tumoricidal activity correlated with reduced expression of mRNAs coding for granzyme B, perforin, Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL). Interleukin-2 (IL-2) and interferon-γ (IFN-γ) synthesis by AK-T cells was also inhibited by adenosine. The inhibitory effect of adenosine on AK-T cell proliferation was also blocked by an A_3 receptor antagonist suggesting that adenosine acts through A_3 receptors to prevent AK-T cell induction. Tumor-associated adenosine may act through the same mechanism to impair the development of tumor-reactive T cells in cancer patients. Therefore the suppression of T-killer cell function suggests that adenosine may act as a local immunosuppressant within the microenvironment of solid tumors. Subsequently it was reported that adenosine partially inhibits the interaction of T lymphocytes with tumor cells by blocking the function of integrin α4β7 which is the major cell adhesion molecule involved in the adhesion of T cells to syngeneic MCA-38 adenocarcinoma cells (MacKenzie et al. 2002). The effect of adenosine has been investigated on the expression of costimulatory molecules by T cells in resting and activated conditions. The most important costimulatory molecules present on the T cells surface are CD2 and CD28 acting in concert to achieve optimal costimulation of T lymphocytes during interaction with antigen presenting cells. It has also been demonstrated that adenosine interferes with activation-induced expression of the co-stimulatory molecules

CD2 and CD28 by an IL-2 dependent mechanism but not involving the accumulation of intracellular cAMP and possibly by activating the A_3 subtype (Butler et al. 2003). Subsequently the inhibitory effect mediated by adenosine on the ability of LAK cells to kill tumor cells was attributed essentially to the cAMP-elevating A_{2A} receptor whilst no evidence of the involvement of cAMP inhibitory A_1 or A_3 subtypes in the regulation of the cytotoxic activity of LAK cells was found (Raskovalova et al. 2005). Indeed, it has been suggested that hypoxic cancerous tissues may be protected by the same hypoxia→adenosine→A_{2A} receptor pathway that was recently shown to be critical and nonredundant in preventing excessive damage of normal tissues by overactive immune cells in vivo (Ohta and Sitkovsky 2001).

In contrast to the immunosuppressive role of adenosine in the environment of solid tumors, it has been reported that A_3 receptor activation stimulates the proliferation of murine bone marrow cells in vitro. This effect was induced through the G-CSF production by human peripheral blood mononuclear cells (PBMC) mediated by adenosine. The finding was confirmed in in vivo experiments, which revealed an increase in leukocyte and neutrophil numbers when adenosine was administered before chemotherapy (Fishman et al. 2000). The molecular mechanisms underlying G-CSF production included the upregulation of the PI3K, PKB/Akt and NF-kB pathways (Bar-Yehuda et al. 2002). In addition, it has been observed that Cl-IB-MECA increases the activity of NK cells in naïve and tumor bearing mice through the induction of IL-12; this effect was dependent on inhibition of cAMP levels and PKA expression. IL-12 is a potent stimulant of NK cells and is a cytotoxic factor that exerts a potent anti-tumor effect in vivo. It induces IFN-γ production by activated T and NK cells and augments cytotoxic activity of these cells via perforin, Fas and Trail-dependent mechanisms. Therefore, A_3 receptor activation enhances NK cell activity and probably NK cell-mediated destruction of tumor cells (Harish et al. 2003). The expression of A_3 receptor was also investigated in resting and activated lymphocytes (Gessi et al. 2004b). Activated human lymphocytes undergo a rapid induction of both transcript and protein of A_3 receptors. The kinetics of this up-regulation revealed that even at earlier time points, the increase was present only in $CD4^+$ cells, whereas it was not changed in $CD8^+$ cells. Therefore, it is possible that in humans, as in mice (Hoskin et al. 2002), A_3 receptors play an immunosuppressive role in $CD8^+$ T cells, but their up-regulation in $CD4^+$ cells strongly suggests that they might also be implicated in T helper cell activities. One method of increasing the number of A_3 receptors on the cell membrane is to increase the accumulation of mRNA encoding the A_3 subtypes. As evaluated by means of real-time RT-PCR experiments, activation of T cells with PHA rapidly increased the level of A_3 message in the $CD4^+$ subset, but not in the $CD8^+$ cells. This increase in A_3 receptor mRNA, which could occur as a result of an increase in transcription and/or an increase in mRNA stability, is likely to be responsible for the increased synthesis of receptor proteins as detected by means of binding and Western blot studies. The rapid up-regulation of A_3 receptors functionally coupled to adenylyl cyclase in activated T cells may indicate another potential example of biological significance for adenosine-mediated responses in T cells.

An overexpression of A_3 receptors has also been detected in lymphocytes of patients with colorectal cancer. Interestingly, the existence of A_3 receptors was previously demonstrated on Jurkat cells, a human leukemic cell line, where they were associated with inhibition of adenylyl cyclase activity and calcium modulation (Gessi et al. 2001). Blood lymphocytes obtained from 30 colorectal cancer patients showed a > threefold overexpression of A_3 receptors compared with blood cells from healthy donors, in line with the data found in tissues. No association was found with stage of the disease, tumor site, patient age, or gender. Even though the mechanism of this up-regulation are not known it is interesting that binding data from tissues, as in circulating blood cells, discriminate between small-sized adenomas and cancer, suggesting that A_3 receptor may be a requirement for colorectal tumor progression. These receptors may represent, like those in neutrophils, tumoral markers due to their higher expression in comparison to that observed in healthy subjects. This suggests that peripheral blood cells mirror at the peripheral level the higher levels of the A_3 receptor found in colorectal cancer. However the selectivity of the A_3 receptor as a tumoral marker may be of only limited value because a similar phenomenon has been confirmed in patients with rheumatoid arthritis. Thus the A_3 receptor was overexpressed in PBMC of patients with rheumatoid arthritis compared to healthy subjects and was directly correlated to an increase in NF-kB in the same cells (Madi et al. 2007). Similar data were found in phytohemagglutinin and lipopolysaccharide-stimulated PBMC from healthy subjects suggesting that receptor upregulation is induced by inflammatory cytokines controlling the expression of the A_3 adenosine receptor transcription factor NF-kB (Madi et al. 2007). It seems that the A_3 adenosine subtype found in PBMC obtained from peripheral blood may not represent a specific tumoral marker but more generally a marker for inflammation.

In conclusion, it is well established that extracellular adenosine has the potential to be an important inhibitor of tumor cell destruction by NK and LAK cells within the microenvironment of solid tumors by signaling primarily through A_{2A} and A_3 adenosine receptors on the surface of T cells (Hoskin et al. 2008). However after the demonstration that genetic deletion of immunosuppressive A_{2A} and A_{2B} receptors or their pharmacological inactivation can prevent the inhibition of anti-tumor T cells by the hypoxic tumor and facilitate full tumor rejection, several reviews focused on the relevance of A_{2A} and in minor part of A_{2B} adenosine subtypes to improve the effectiveness of immune-based cancer therapies (Ohta et al. 2006; Lukashev et al. 2007; Sitkovsky et al. 2008a, b). In contrast to the well described mechanisms by which A_{2A} adenosine receptor signaling blocks T cell activation and effector function, little is known about the mechanism of A_3 adenosine receptor-mediated T cell inhibition. Moreover, while the importance of A_{2A} adenosine receptor signaling in adenosine-mediated suppression of T cell responses has been confirmed using A_{2A} adenosine receptor-deficient mice (Lukashev et al. 2003), similar confirmatory studies have not yet been performed with A_3 adenosine receptor deficient mice. Additional studies need to be performed in human lymphocytes as almost all the functional effects attributed to A_3 receptor activation are derived from studies carried out in mice species (Fig. 12.3). The identification of adenosine

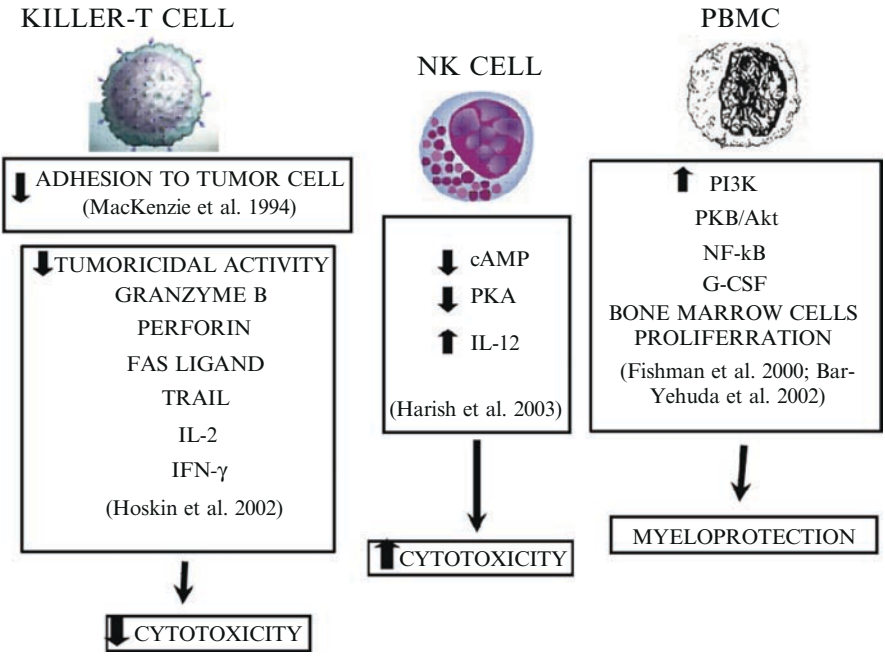

Fig. 12.3 Effects of A_3 adenosine receptors in lymphocytes. Given the important species differences in the location and functional response to A_3 receptors, only the effects obtained in human are in *bold*

receptor subtypes and/or signal transduction pathways through which adenosine exerts its inhibitory effects on cell-mediated anti-tumor immune responses may allow for the development of novel "anti-adenosinergic" approaches that increase the effectiveness of therapeutic cancer vaccines and other immune-based cancer therapies.

12.4 A_3 Adenosine Receptor Effects on Monocyte-Macrophage Function

In vivo and in vitro studies in animal systems led to the concept of the mononuclear-phagocyte system as a cell system involved in host defenses, phagocytosis, and antigen presentation and processing (Douglas 1999). Following Metchnikoff's development of phagocyte theory, Wright described opsonins as factors in serum that facilitated phagocytosis. Aschoff defined the reticuloendothelial system as a cellular system in which tissue macrophages and monocytes share important functional characteristics, namely, phagocytic ability and adhesiveness to glass. Subsequently, the histologic development of silver stains by Del Rio-Hortega defined a type of macrophage-related cell in the brain, the microglia. In the mid-

1960s, the late Zanvil Cohn and his collaborators carried out seminal studies of mononuclear phagocytes leading to concepts of macrophage differentiation, activation, secretion, and the relationship of macrophages to antigen presentation and processing (for further details see Douglas 1999).

Adenosine has been investigated as an endogenous regulator of monocyte-macrophage functions. The effects produced by A_3AR activation of macrophages seem to indicate an anti-inflammatory effect of this adenosine subtype. For example, the A_3ARs suppress TNF-α release induced by the endotoxin CD14 receptor signal transduction pathway from human monocytes and murine J774.1 macrophages (Le Vraux et al. 1993; McWhinney et al. 1996). Moreover in a macrophage model the A_3AR was the prominent subtype implicated in the inhibition of LPS-induced TNF-α production (Sajjadi et al. 1996). This effect was associated with changes in stimulation of the activator protein-1 (AP-1) transcription factor, whereas it was independent of MAPKs, NF-kB, PKA, PKC and PLC. The inhibitory effect induced by the A_3AR on TNF-α production was also assessed in A_3KO mice where the A_3 agonist was unable to reduce TNF-α levels in contrast to its effect in wild type animals (Salvatore et al. 2000). In contrast, in BV2 microglial cells the A_3-mediated inhibition of LPS-induced TNF-α expression was associated with the inhibition of LPS-induced activation of the PI3-kinase/Akt and NF-kB pathways (Lee et al. 2006). Recently it has been reported that in mouse RAW 264.7 cells the A_3 subtype inhibits LPS-stimulated TNF-α release by reducing calcium-dependent activation of NF-kB and ERK 1/2 (Martin et al. 2006). In contrast, in peritoneal macrophages, isolated from A_3 KO mice, the ability of IB-MECA to inhibit TNF-α release was not altered in comparison to wild type mice (Kreckler et al. 2006). In this study, the inhibitory effect was exerted through the activation of A_{2A} and A_{2B} agonists as has been recently demonstrated in human monocytes (Zhang et al. 2005; Haskó et al. 2007). The discrepancy observed among these papers cannot be the consequence of species differences, since in both cases mouse cells were used. Other factors, including the source of the cells and/or the inflammatory stimulus used, may be responsible. However in spite of these contrasting results, one of the most likely therapeutic applications of the regulatory role of A_3 activation on TNF-α release is in the treatment of arthritis. More recent studies show that A_3AR agonists exert significant effects in different autoimmune arthritis models by suppression of TNF-α production (Baharav et al. 2005). The molecular mechanisms involved in the inhibitory effect of IB-MECA on adjuvant-induced arthritis include receptor downregulation and de-regulation of the PI3K-NF-kB signalling pathway (Fishman et al. 2006; Madi et al. 2007). Thus, A_3AR activation by IB-MECA inhibited macrophage inflammatory protein (MIP)-1α, a C-C chemokine with potent inflammatory effects, in a model of collagen-induced arthritis, providing the first proof of concept of the adenosine agonists utility in the treatment of arthritis (Szabo et al. 1998). Other anti-inflammatory effects involving A_3 receptors activation include inhibition of fMLP-triggered respiratory burst and tissue factor expression by human monocytes (Broussas et al. 1999, 2002). Recently, it has been reported that, adenosine may be involved in ventricular remodeling by stimulating Matrix metalloproteinase-9 (MMP-9) production in human

macrophages following A_3 receptor activation (Velot et al. 2008). MMP-9 plays an important role in ventricular remodelling after acute myocardial infarction (MI). Adenosine enhanced MMP-9 production when macrophages were activated by hypoxia or Toll-like receptor-4 ligands such as lipopolysaccharide, hyaluronan, and heparan sulfate. The effect of adenosine was replicated by the A_3 agonist IB-MECA and inhibited by silencing the A_3AR through the use of RNA interference. Interestingly, it was found that MMP-9 expression was higher in blood cells from patients with acute MI compared with healthy volunteers with important implications for therapeutic strategies targeting adenosine receptors in the setting of MI (Velot et al. 2008).

In conclusion as for the role of A_3 receptors in the inhibition of TNF-α production in macrophages discrepant results have been obtained and not only due to the different species considered. For example some studies attributed reduction of TNF-α to A_3 receptors either in human and mouse species (Sajjadi et al. 1996; McWhinney et al. 1996), whilst other found this effect to be mediated essentially by A_{2A} and in minor part by A_{2B} without the involvement of the A_3 receptors again in both human and mouse species (Zhang et al. 2005; Kreckler et al. 2006). Therefore it is difficult in this case to verify the relevance of the A_3 receptor-induced cellular response when other adenosine subtypes like A_{2A} and A_{2B} are also activated. As for the effects exerted by the A_3 subtype in human monocytes and macrophages it is possible to find support for an anti-inflammatory role for this receptor as attested by reduction of tissue factor, oxidative burst and perhaps TNF-α release. Also the recent discovery of an increase in MMP9 supports a role for A_3 agonists in the therapy of myocardial infarction (Velot et al. 2008) (Fig. 12.4).

12.5 A_3 Adenosine Receptor Effects on Dendritic Cell Function

Dendritic cells are antigen-presenting cells specialized to activate naive T lymphocytes and initiate primary immune responses (Steinman 1991; Hart 1997; Banchereau and Steinman 1998). Dendritic cells originate from hemopoietic stem cells and migrate into peripheral tissues. Dendritic cells reside in an immature form in unperturbed tissue, where they are capable of taking up antigens but weak at stimulating T cells. Under the influence of a variety of so-called danger signals including pathogens; dying cells; soluble CD40 ligand; cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6), or bacterial products such as LPS dendritic cells undergo a process of differentiation known as maturation. Thereafter, they migrate to the T-cell areas of secondary lymphoid organs. This maturation process is associated with reduced phagocytic and endocytic activity, increased membrane expression of major histocompatibility complex and co-stimulatory molecules, production of cytokines such as interleukin 12 (IL-12), and acquisition of potent T-cell-stimulating functions. Depending on the conditions, dendritic cells can stimulate growth of a variety of T-cell subsets. In the presence of IL-12, they support the growth of Th1 cells, whereas with IL-4 dendritic cells induce Th2-cell

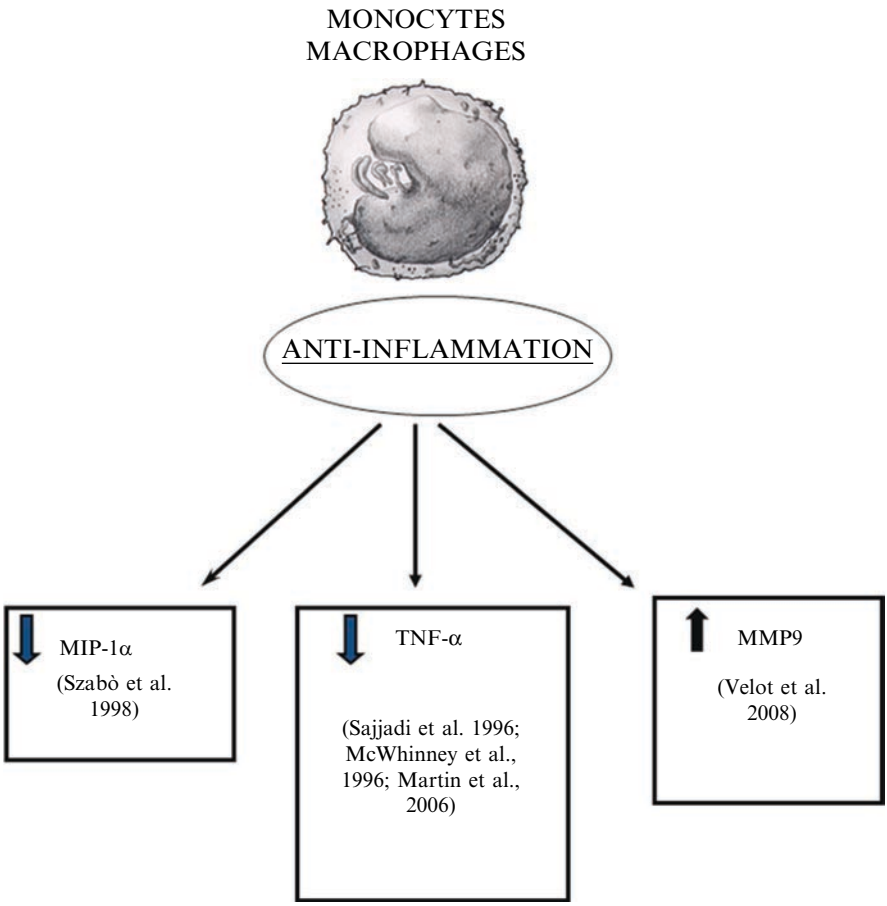

Fig. 12.4 Effects of A_3 adenosine receptors in monocytes-macrophages. Given the important species differences in the location and functional response to A_3 receptors, only the effects obtained in human are in *bold*

differentiation. In recent years it has become clear that A_3 adenosine receptors play a role in regulation of various activities of dendritic cells.

The expression and function of adenosine receptors in human dendritic cells has been investigated by using reverse transcriptase-polymerase chain reaction and functional experiments (Panther et al. 2001). mRNA expression of the A_3 receptor has been detected in immature dendritic cells together with A_1 and A_{2A} receptors. Adenosine, IB-MECA and also the A_1 agonist CHA, induced Ca^{2+} transients as well as actin polymerization and chemotaxis but only in immature dendritic cells. These findings suggest that adenosine may control proinflammatory activities of dendritic cells and regulate their accumulation at target sites. Maturation of dendritic cells is accompanied by a loss of the adenosine responses such as Ca^{2+} transients, actin

polymerization, and migration. Unequivocal evidence of cell surface expression of the A_3 receptor in immature dendritic cells was obtained from [^{125}I]ABMECA binding experiments. Saturation isotherms indicated a Bmax of approximately 300 fmol/mg membrane protein, and competition for the radioligand of a variety of adenosine receptor ligands categorically identified the binding site as the A_3 receptor (Fossetta et al. 2003). Moreover through fluorometric imaging plate reader (FLIPR)-based analysis of calcium mobilization it was shown that the A_3 adenosine receptor is coupled to calcium mobilization in a pertussis toxin- dependent way. Interestingly these authors demonstrated that adenosine is much more potent at the A_3 receptor than had been appreciated, being active in the low nanomolar range. Generally, adenosine has been regarded as a low potency agonist of the A_3 receptor, with apparent affinities ranging from 300 nM to 1 μM (Fredholm et al. 2001). The presence of functional A_3 receptors has been observed in XS-106, a mouse dendritic cell line, where they were coupled negatively to adenylyl cyclase and to stimulation of p42/p44 mitogen-activated protein kinase phosphorylation. Adenosine A_3 receptor activation also inhibits lipopolysaccharide-induced TNF-α release from XS-106 cells as already reported in macrophages (McWhinney et al. 1996; Dickenson et al. 2003). At present, the signal transduction pathway involved in adenosine A_3 receptor-mediated inhibition of TNF-α release from XS-106 cells (and see above macrophages) is unclear. Inhibition of TNF-α release is usually associated with Gs-protein-coupled receptor-mediated cyclic AMP production. Interestingly, adenosine A_3 receptors have been shown to induce an increase in intracellular calcium and potentiate Ca^{2+} currents via protein kinase A activation in A6 renal cells (Reshkin et al. 2000) and hippocampal CA_3 pyramidal neuronal cells (Fleming and Mogul 1997). In addition, activation of the adenosine A_3 receptor stimulates cyclic AMP production in human eosinophils (Ezeamuzie and Philips 2003). However, in XS-106 cells, Cl-IB-MECA did not stimulate cyclic AMP accumulation indicating that the adenosine A_3 receptor is not directly coupled to Gs-protein/cyclic AMP accumulation in XS-106 cells. Finally, the transcript for the A_3 adenosine receptor was elevated more than 100-fold in immature dendritic cells compared with monocyte precursors. A_3 receptor transcript was substantially diminished by LPS-induced maturation of immature dendritic cells. The strict dependence of A_3 receptor expression on the immature cells suggests that the A_3 receptor could also be involved in the maintenance of the immature phenotype, and its abrupt disappearance may be crucial for transition to a fully activated dendritic cell (Fossetta et al. 2003).

The relevance of the A_3 receptor over the other adenosine subtypes in immature human dendritic cells is attested to by different studies demonstrating a role for this receptor in the increase of intracellular calcium, actin polymerization and chemotaxis (Panther et al. 2001; Fossetta et al. 2003) (Fig. 12.5). However a loss of the A_3 and an increase of the A_{2A} receptor has been reported during maturation of dendritic cells. This switch has been interpreted as a protective effect of adenosine in the context of tissue injury as A_{2A} activation plays an inhibitory role on dendritic cells migration. In this way adenosine could counterbalance inflammatory stimuli by delaying the arrival of mature dendritic cells to lymph nodes, thereby impairing the

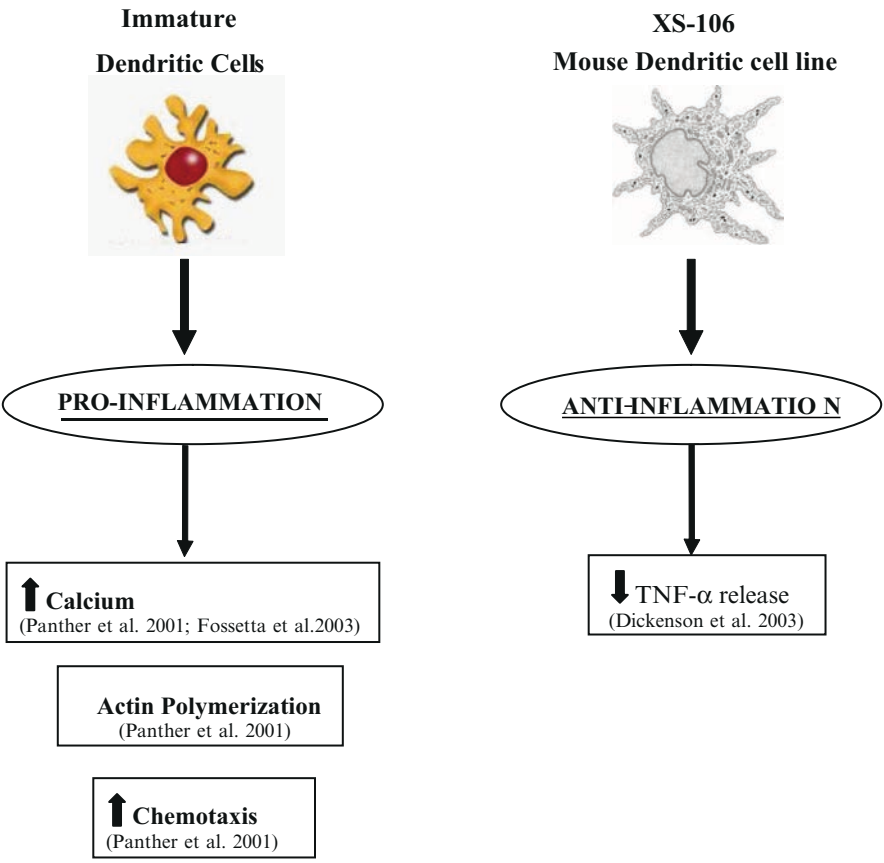

Fig. 12.5 Effects of A_3 adenosine receptors in dendritic cells. Given the important species differences in the location and functional response to A_3 receptors, only the effects obtained in human are in *bold*

initiation of immune responses and reducing the potentially detrimental effects of chronic cell activation responsible for tissue damage and disease.

12.6 Conclusions

The data summarized in this chapter show that A_3 receptors are present in immune cells and are indeed involved in the physiopathologic regulation of inflammatory and immune processes. However results from in vitro and in vivo studies in experimental animals suggest activation of the A_3 subtype can be both pro or anti-inflammatory depending on:

1. The cell type examined, for example neutrophil, eosinophil, macrophage, T cell, dendritic cell
2. The cellular model used, for example in vitro or ex vivo; transgenic animals
3. The response investigated, for example degranulation, oxidative burst, migration, maturation, cytokine production
4. The species considered, for example human or animal
5. The presence and functional roles of other adenosine receptor subtypes

Even though it seems that in each cell type examined contrasting effects have been reported, the results reviewed here offer the background for possible new therapeutic strategies for a number of inflammatory conditions such as sepsis, asthma and autoimmune disorders including rheumatoid arthritis, Crohn's disease and psoriasis. Indeed at the moment there are A_3AR agonists in clinical development for rheumatoid arthritis. Unfortunately there are no A_3AR antagonists in clinical development but a number of molecules are in biological testing as therapeutic agents for asthma and COPD, glaucoma, and stroke, waiting to enter the clinical arena (Baraldi et al. 2008). Future studies aimed at elucidating new effects of the A_3 subtype in the modulation of important inflammatory responses in the different peripheral blood cells are likely to reveal exciting new potential therapeutic applications of A_3 agonists and/or antagonists.

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Chapter 13
Adenosine A_3 Receptors in Muscle Protection

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13.1 Biological Models and Relevant Pharmacology of Adenosine A_3 Receptor Agonists and Antagonists

Given the intense interest on adenosine and adenosine receptor-induced cytoprotective effects, a number of biological models have been developed to study the role of each receptor subtype and to investigate its underlying signaling mechanism. Historically, research on the protective action of adenosine A_3 receptors began with protection of the heart from ischemia/reperfusion injury. A growing body of evidence has accumulated to support a cardio-protective effect of adenosine A_3 receptor activation. This topic is well-addressed in another chapter in the book, only a brief summary will be described when relevant.

Of particular interest is a proof of principle study showing that cardiac-specific overexpression of the A_3 receptor can protect the murine myocardium against ischemia/reperfusion (Cross et al. 2002; Black et al. 2002). Although some raised the question that the widely used prototypical agonist Cl-IBMECA (2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide) may not be selective at the adenosine A_3 receptor in intact heart, Cl-IBMECA could not exert a cardio-protective effect in A_3R-knockout mice, pointing to an essential role of the A_3 receptor in

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mediating the cardio-protective effect of Cl-IBMECA (Ge et al. 2006). The role of the adenosine A_3 receptor was further extended to non-rodent mammalian models. Thus, Cl-IBMECA was able to reduce myocardial ischemia–reperfusion injury in both rabbits and dogs (Auchamapach et al. 1997, 2003).

13.1.1 Skeletal Muscle Protection

Ischemia and reperfusion can cause significant injury of skeletal muscle, which is the most vulnerable tissue in the extremities (Blaisdell 2002; Ecker and Schnackerz 1991). Trauma, autogenous skeletal muscle transplantation, surgical incision, vascular clamp application during vascular surgery or musculoskeletal reconstructive surgery as well as sustained strenuous exertion can also induce skeletal muscle damage with deleterious systemic consequences (Blaisdell 2002; Beyersdorf et al. 1991; Carrol et al. 1997). Protection of skeletal muscle from ischemia and reperfusion injury is therefore an important therapeutic goal directed toward ameliorating muscle and organ injury. Although various measures such as a tissue-preserving solution and cold immersion are used to preserve intact organs and skeletal muscle (Gallin and Snyderman 1999; Southard and Belzer 1995; Tsuchida et al. 2003), an effective method or agent to protect skeletal muscle from ischemia/reperfusion injury is lacking. Because adenosine and ischemic preconditioning can provide potent protection of the heart muscle, interest is emerging to study whether ischemic preconditioning and adenosine receptors can also induce protection of the skeletal muscle.

Four consecutive 5-min periods of ischemia separated by 5-min reperfusion periods or intravenous injection of adenosine prior to 60 min of suprarenal aortic clamping and 30-min reperfusion resulted in the preservation of contractile function and ATP/creatine phosphate levels (Lee et al. 1996). Other investigators investigated whether acute ischemic preconditioning can protect pig skeletal muscle from ischemia–reperfusion injury (Pang et al. 1995, 1997; Moses et al. 2005). Using a global ischemia–reperfusion model in the pig latissimus dorsi and gracilis muscles, at least three cycles of 10 min ischemia and 10 min reperfusion were needed to reduce infarct size. Concomitant with infarct size reduction, the muscle content of ATP, phosphocreatine and energy charge potential in the preconditioned skeletal muscle were significantly higher than in the nonpreconditioned muscle. Thus, in both rodent and non-rodent species, ischemic preconditioning was associated with a prominent energy sparing effect in the skeletal muscle. Whether energy sparing is a mechanism of preconditioning in skeletal muscle remains to be determined.

Similar to cytoprotection of the heart, adenosine is implicated in mediating the protective effect of preconditioning in skeletal muscle (Pang et al. 1997; Bushell et al. 2002a, b). Direct infusion of adenosine can mimic the skeletal muscle protective effect of ischemic preconditioning in extensor digitorum longus muscle before aorta occlusion in the rat as well as in the pig latissimus dorsi

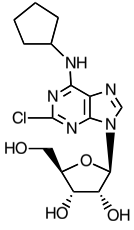
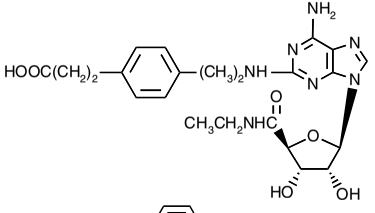
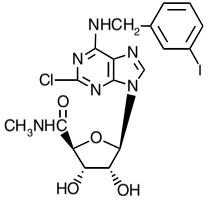
muscle flap model. While these initial data suggest an important regulatory role of adenosine, identity of the adenosine receptor subtypes mediating skeletal muscle protection and their underlying signaling mechanism are not well understood.

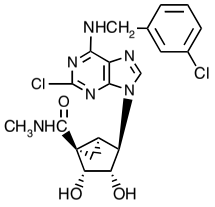
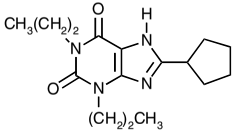
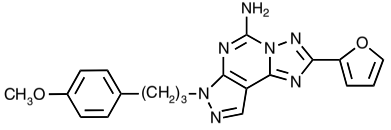
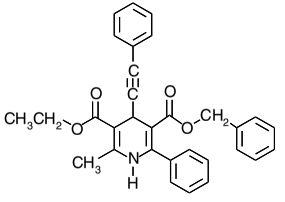
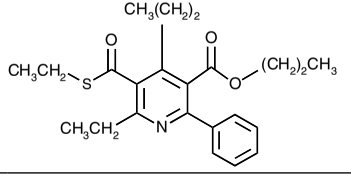
N^6-(R-phenyl-2-propyl)adenosine (R-PIA), an adenosine A_1 receptor-agonist of low selectivity, could exert an anti-ischemic effect in a pig latissimus dorsi muscle flap model (Pang et al. 1997). The adenosine A_1 receptor-selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), blocked the protection by adenosine in this model. While these data implicated a role for the adenosine A_1 receptor in mediating the protection against ischemia/reperfusion injury in skeletal muscle, whether other adenosine receptor subtypes can also protect skeletal muscle is unknown. While activation of the adenosine A_3 receptor has been shown to protect the myocardium against ischemia and reperfusion injury (Auchampach et al. 2003; Liang and Jacobson 1998; Maddock et al. 2002), rodent mast cells express the adenosine A_3 receptor whose activation can stimulate inflammation, causing a potentially deleterious effect on skeletal muscle (Ramkumar et al. 1993; Tilley et al. 2000). A systematic investigation of the cytoprotective role of adenosine A_1, A_{2A} and A_3 receptors in skeletal muscle is lacking. A genetic approach as well as a full pharmacological characterization using selective agonists and antagonists will provide a more comprehensive answer to this important question.

13.1.2 Pharmacology of Adenosine Receptors and Relevance to Skeletal Muscle Protection

Subtype-selective agonists and antagonists of the adenosine receptors have been developed (Jacobson and Gao 2006). Table 13.1 shows the affinity at the human subtypes of selective ligand probes of the A_1, A_{2A}, and A_3 receptors used in skeletal muscle studies. Two agonists of the A_3 receptor are listed: Cl-IBMECA is a 9-riboside derivative like native adenosine, and MRS3558 ((1′S,2′,3′S,4′R,5′S)-4′-{2-chloro-6-[(3-chlorophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0] hexane-2,3-diol) is a newer selective agonist based on replacement of the native ribose ring with a conformationally rigid (N)-methanocarba ring system. This ring system of MRS3558 closely approximates the optimal conformation needed for recognition in the A_3 receptor, which accounts for its increased affinity. This compound has been shown to be anti-inflammatory in a model of adjuvant induced arthritis in rats (Ochaion et al. 2008). SCH 442416 (5-amino-7-(3-(4-methoxy) phenylpropyl)-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) is a recently introduced and highly potent and selective A_{2A} receptor antagonist, and its use as an *in vivo* imaging agent has been demonstrated (Moresco et al. 2005). MRS1523 (5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate) is a moderately selective A_3 receptor antagonist in several species. MRS1523 binds to human adenosine A_1 receptors with a $K_i > 10{,}000$ nM and to human A_{2A} receptors with a K_i of 3,660 ± 930 nM (±SEM).

Table 13.1 Structures and addinities at four subtypes of adenosine receptors of selective ligands used in studying skeletal muscle protection.

Compound	Structure	Affinity (K_i, nM) Human[a] A_1	Human[a] A_{2A}	Human[a] A_{2B}	Human[a] A_3
Adenosine receptor agonists					
CCPA		0.83[b] 1.3 (r)[c] 0.1 (rb)[d]	2270[b] 950 (r)[c]	18,800[b]	38[b] 237 (r)[c] 37.7 (rb)[d]
CGS21680		289[b] 1800 (r)[c] 120 (rb)[d]	27[b] 19 (r)[c]	>10,000[b] >10,000 (r)[c]	67[b] 584 (r)[c] 673 (rb)[d]
CI-IBMECA		220[b] 280 (r)[c] 35 (m)[c]	5360[b] 470 (r)[c] ~10,000 (m)[c]	>10,000[b] >10,000 (m)[c]	1.4[b] 0.33 (r)[c] 0.18 (m)[c]

MRS3558		260[b] 105 (r)[f] 15.8 (m)[c]	2300[b] 1080 (r)[f] 10,400 (m)[c]	>10,000[b]	0.29[b] 1.0 (r)[f] 1.49 (m)[c]
Adenosine receptor antagonists					
DPCPX		3.9[b] 0.46 (r)[c] 0.29 (c)[g] 3.9 (gp)[g] 0.68 (rb)[d]	129[b] 340 (r)[c]	63.8[h] 185[h]	3980[b] 5290 (r)[c] 5270 (rb)[d]
SCH 442416		1110[b]	0.048[b]	>10,000[b]	>10,000[b]
MRS1191		>10,000 40,100(r) 16% (1μM,rb)[d]	>10,000 >10,000(r)	>10,000	31.4 1850 (r)[i] 54% (1μM,rb)[d]
MRS1523		>10,000 15,600(r)[b] 8000 (m)[j]	3660 ± 930 2050 (r)[b] >10,000(m)[j]	>10,000 >10,000 (m)[j]	18.9[b] 113 (r) 731 (m)[j]

(continued)

Table 13.1 (continued)

[a]Human, unless other species is noted., c = calf; gp = guinea pig; r = rat; rb = rabbit; m = mouse. A percent refers to percent of radioligand binding at the indicated concentration.

[b]Jacobson and Gao 2008.

[c]Müller CE. Adenosine Receptor Ligands–Recent Developments Part I. Agonists. *Current Medicinal Chemistry* 7, 1269–1288, 2000.

[d]Takano H, Roberto Bolli R, Richard G, Black RG, Eitaro Kodani E, Xian-Liang Tang XL, Yang Z, Bhattacharya S, Auchampach JA. A_1 or A_3 Adenosine Receptors Induce Late Preconditioning Against Infarction in Conscious Rabbits by Different Mechanisms. *Circ. Res*. 88, 520–520, 2001.

[e]Melman et al. 2008.

[f]Ochaion et al. 2008.

[g]Ukena D, Jacobson KA, Padgett WL, Ayala C, Shamin MT, Kirk KL, Olsson RA, Daly JW, Species differences in structure-activity relationships of adenosine agonists and xanthine antagonists at brain A1 adenosine receptors *FEBS Lett*. 209, 122–128, 1986.

[h]Kim SA, Marshall MA, Melman N, Kim HS, Müller CE, Linden J, Jacobson KA. Structure activity relationships at human and rat A_{2B} adenosine receptors of xanthines substituted at the 1-, 3-, 7-, and 8-positions. *J. Med. Chem*. 45, 2131–2138, 2002.

[i]Gao ZG, Teng B, Wu H, Joshi BV, Griffiths GL, Jacobson KA. Synthesis and pharmacological characterization of [^{125}I]MRS1898, a high affinity, selective radioligand for the rat A_3 adenosine receptor. Purinergic Signalling, doi: 10.1007/s11302-008-9113-3.

[j]Ge ZD, Peart JN, Kreckler LM, Wan TC, Jacobson MA, Gross GJ, Auchampach JA. ChIB-MECA [2-chloro-*N*^{6}-(3-iodobenzyl)adenosine-5′-*N*-methylcarboxamide] reduces ischemia/reperfusion injury in mice by activatating the A_3 adenosine receptor. *J Pharmacol. Exp. Ther.* 319, 200–1210, 2006.

Several caveats are to be noted in connection with the use of these agents. The selectivity is not absolute and the affinities can vary among species. For example, the A_1 agonist CCPA (2-chloro-N^6-cyclopentyladenosine) also has considerable affinity at the human A_3 receptor, at which it can act as an antagonist (Gao et al. 2002). The agonist CGS21680 (2-[p-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine) is more selective for the A_{2A} receptor in the rat than in human tissue, and it binds appreciably at the A_3 receptor. The selectivity of MRS3558 for the A_3 receptor is greater in human than in mouse tissue (Melman et al. 2008). The A_1 receptor antagonist DPCPX, like the prototypical nonselective adenosine receptor antagonists caffeine and theophylline, is a xanthine derivative that also displays considerable affinity at the A_{2B} receptor. Also, the affinity of the A_3 receptor antagonist MRS1191 (1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl 5-(phenylmethyl) ester) is much lower in murine species than in human, although its selectivity at the A_3 receptor remains. Finally, the potency of agonists can vary considerably depending on the receptor expression level. For these reasons, it is recommended to use multiple agonists and antagonists of diverse chemical structure in order to conclude that a particular adenosine receptor subtype is involved in a given pharmacological action.

13.2 Role of Adenosine and Adenosine Receptor Subtypes in Muscle Protection

Using a murine model of hindlimb ischemia–reperfusion injury, we (Zheng et al. 2007) studied the anti-ischemic role of various adenosine receptor subtypes. The injury was created by placement of a constrictor band above the greater trochanter with a McGiveney Hemorrhoidal Ligator. After 90 min of warm ischemia at 37°C, the constrictor was removed to allow reperfusion for 24 h, at which time muscle injury was determined. In this model, two independent methods were used to quantify the extent of skeletal muscle injury. The first method used Evans Blue dye (EBD) to stain for injured skeletal muscle. EBD was bound to serum albumin and could only get inside a cell when its membrane integrity was disrupted during ischemia. Quantifying the EBD-positive area of skeletal muscle section provided a useful index of muscle injury. Another method relied on measurement of serum creatine kinase (CK) activity. CK is released from injured skeletal muscle into the circulation. Its activity can be determined and can serve as an index of skeletal muscle injury.

In this murine model, adenosine A_1 receptors could induce protection against ischemia–reperfusion injury of the hindlimb skeletal gastrocnemius muscle (Fig. 13.1). The protection by A_1 receptor-selective agonist CCPA, administered 2 h before the induction of ischemia, was completely abrogated by the A_1 antagonist DPCPX but was insensitive to blockade by the A_3 receptor antagonist MRS1191. These data confirmed an important role of the adenosine A_1 receptor in mediating an anti-ischemic effect in the skeletal muscle. Extending the traditional pharmacological approach to defining the function of other adenosine receptor subtypes, the role of adenosine

A_3 receptors in affording skeletal muscle protection was investigated. The A_3 receptor-selective agonist Cl-IBMECA exerted a potent reduction of skeletal muscle injury when it was administered before the onset of ischemia. The reduced skeletal muscle injury was reversed by the presence of the A_3 receptor-selective antagonist MRS1191 but not by that of DPCPX (Fig. 13.2). These data demonstrated for the first time a protective function of the A_3 receptor in skeletal muscle.

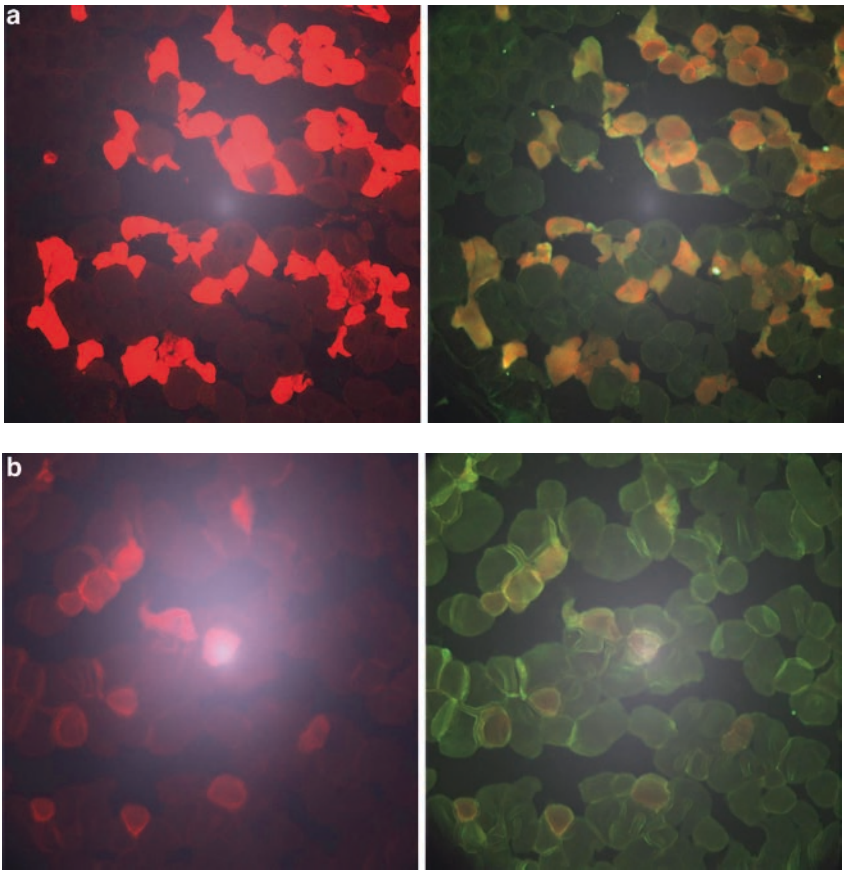


Fig. 13.1 Cytoprotective action of adenosine in a quantitative model of mouse hindlimb ischemia and reperfusion (I/R) injury. Adult wild type mice were injected with (**a**) sterile vehicle (0.1% DMSO in phosphate-buffered saline, pH 7.4) or various adenosine receptor agonists (**b**, **c**) or antagonist (**d**, **f**). (**a**) Following ischemia and reperfusion, skeletal muscle showed a significant uptake of Evans Blue dye (EBD) in vehicle-treated mice (first part of (**a**), representative of 7 mice). The contra-lateral leg not subjected to ischemia–reperfusion showed virtually no EBD uptake. In the second part of (**a**), the same section was stained with rabbit polyclonal anti-skeletal muscle actin antibodies followed by staining with goat anti-rabbit IgG conjugated with FITC. (**b**) The nonselective adenosine agonist R-PIA caused a large reduction in the EBD-stained area (see both first and second parts to this figure, representative of six mice)

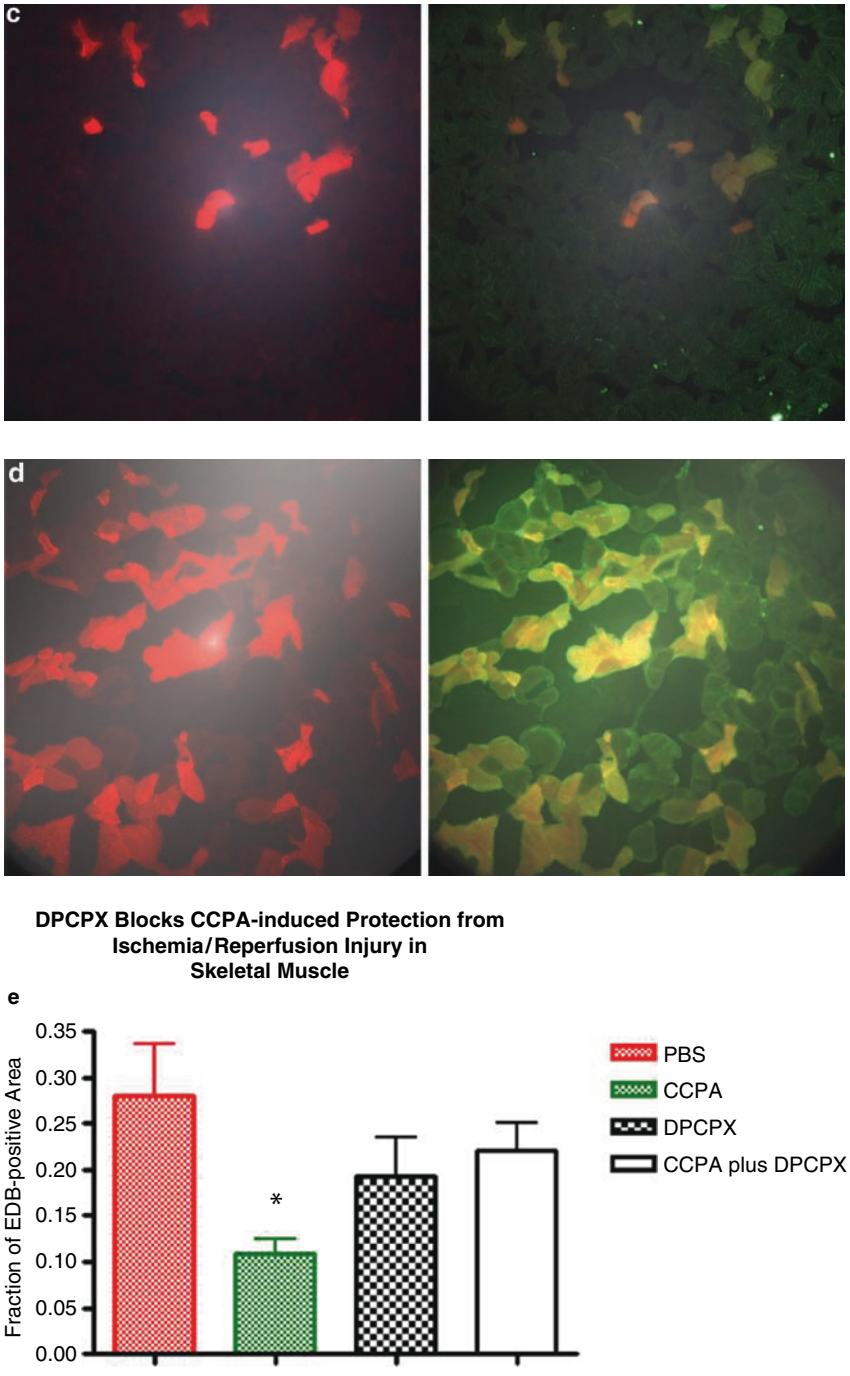


Fig. 13.1 (continued) (**c**) A highly A_1 receptor-selective agonist CCPA decreased the %EBD-positive area (representative of 12 mice); (**d**) this reduction was reversed in mice injected with an A_1 receptor-selective antagonist DPCPX before CCPA (nine mice). (**e**): Average EBD staining (±SD) of skeletal muscle sections following treatment with vehicle- or adenosine ligand were quantified by blinded observers (n = 8 mice for DPCPX alone). *P < 0.05 CCPA vs. PBS, DPCPX or CCPA plus DPCPX

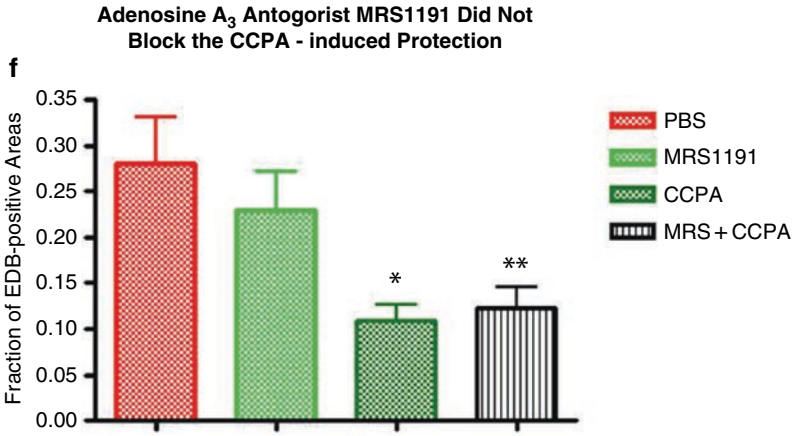


Fig. 13.1 (continued) (**f**): Conversely, MRS1191 could not block the CCPA-induced protection (n = 23 mice for MRS1191 plus CCPA; n = 15 mice for MRS1191 alone). *P < 0.05, CCPA vs. PBS or MRS1191; **P < 0.05, MRS1191 + CCPA vs. PBS or MRS1191 alone. All adenosine receptor ligands were administered intraperitoneally. MRS1191: 0.05 mg/kg; CCPA: 0.05 mg/kg; R-PIA: 0.05 mg/kg; DPCPX: 0.2 mg/kg

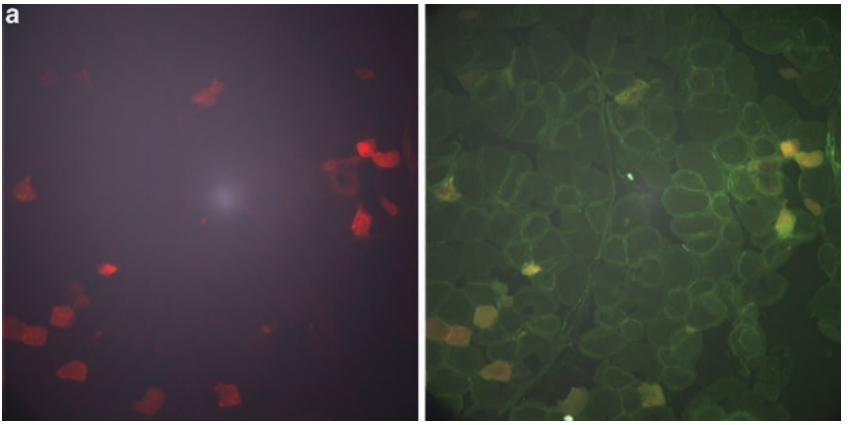


Fig. 13.2 Adenosine A_3 receptors are capable of mediating a potent anti-ischemic protective response distinct from that mediated by the A_1 receptor. (**a**) Adult WT mice injected with an A_3 receptor-selective agonist Cl-IBMECA exhibited a significant reduction in the % EBD-stained muscle (eight mice). The first part of the figure corresponded to EBD-stained muscle while the second part was FITC-stained muscle (**b**) and (**d**) This protection was reversed by the sequential injection of an A_3 receptor-selective antagonist MRS1191 and of Cl-IBMECA (22 mice). *P < 0.05, Cl-IBMECA vs. PBS, MRS1191 or MRS1191 + Cl-IBMECA. (**c**) and (**e**) However, the injection of the A_1 receptor-selective antagonist DPCPX did not prevent the Cl-IBMECA-evoked protection (nine mice). *P < 0.05 Cl-IBMECA vs. PBS or DPCPX; **P < 0.05, DPCPX + Cl-IBMECA vs. PBS or DPCPX. Thus, the protective effect of CCPA and Cl-IBMECA is mediated specifically by its respective adenosine receptors (**d** and **e**): Average EBD staining (±SD) of skeletal muscle sections following treatment with vehicle- or adenosine ligand were quantified by blinded observers (**f**) Using serum creatine kinase (CK) as a marker of muscle injury, the A_3 receptor agonist Cl-IBMECA caused a reduction in the serum CK when the agonist was administered in a manner identical to that applied in legend to Fig. 13.1 and (**a**) (one-way ANOVA and posttest comparison, P < 0.05).

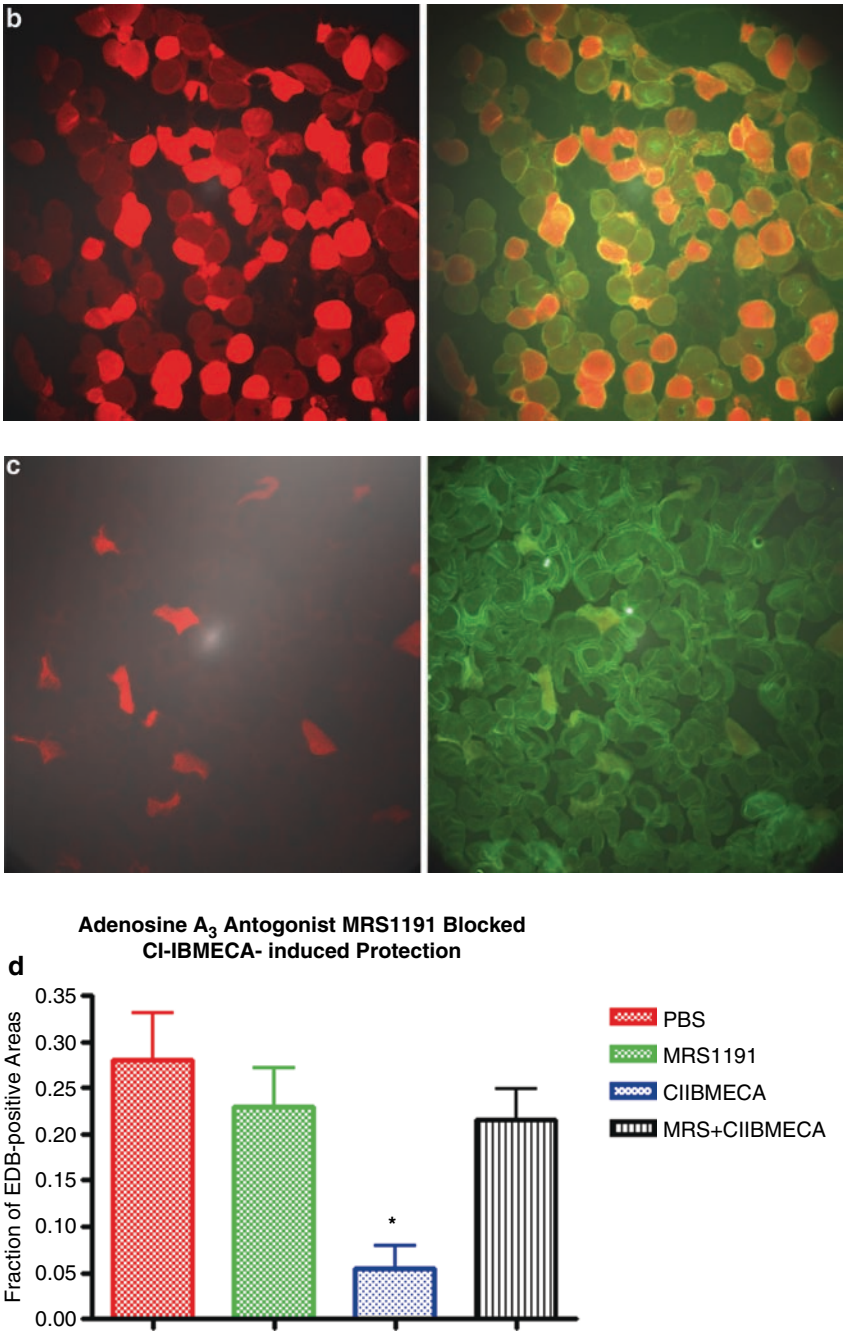


Fig. 13.2 (continued) Similarly, prior treatment with the A_1 receptor agonist CCPA also reduced the serum CK ($P < 0.05$). Data were mean and standard error of 14 mice (PBS-treated), 13 mice (Cl-IBMECA-treated), and 10 mice (CCPA-treated). Cl-IBMECA: 0.07 mg/kg; the other adenosine receptor ligands were administered at dosage described in figure legend of Fig. 13.1

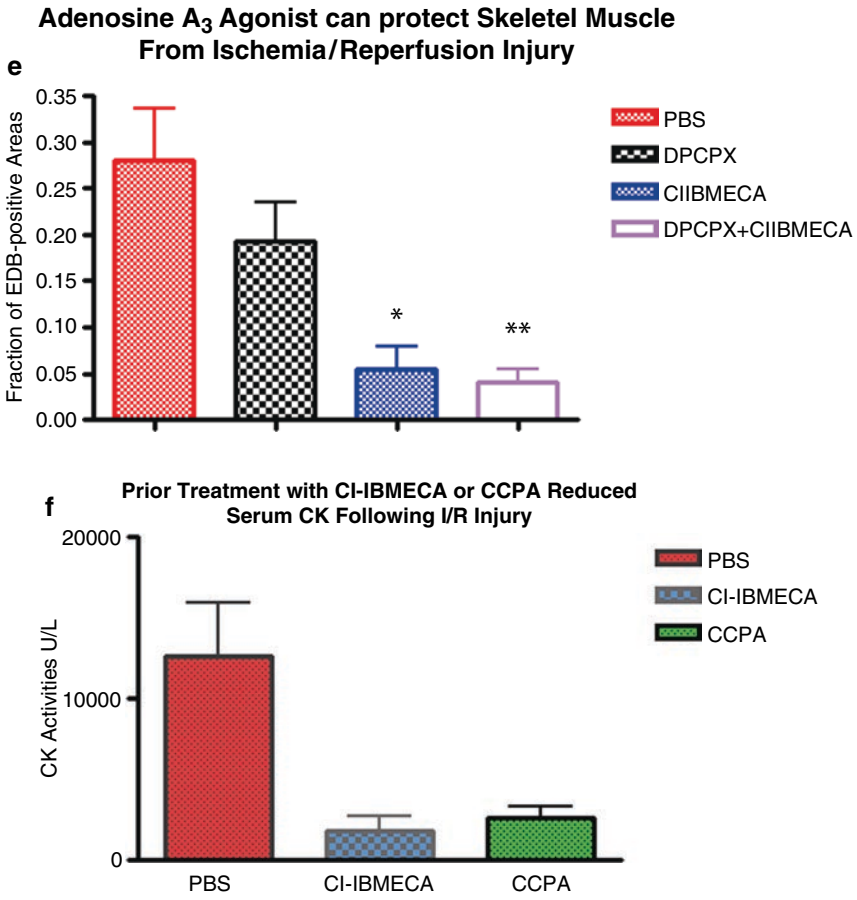


Fig. 13.2 (continued)

In examining the role of adenosine A_{2A} receptors in protecting skeletal muscle from ischemia–reperfusion injury, the model determined the effect on muscle injury in the presence of the A_{2A} receptor-selective agonist CGS21680. CGS21680 could induce a significant reduction of injury when it was administered before the onset of ischemia. This skeletal muscle protective effect was blocked by the A_{2A} receptor-selective antagonist SCH442416 but not by MRS1191 (Fig. 13.3), pointing to an A_{2A} effect.

Taken together, using a mouse model of hindlimb skeletal muscle injury, all three adenosine receptor subtypes could each individually induce protection from ischemia–reperfusion injury in skeletal muscle. Due to the lack of a highly selective A_{2B} receptor agonist and antagonist, its role in mediating an anti-ischemic effect in skeletal muscle remains to be determined.

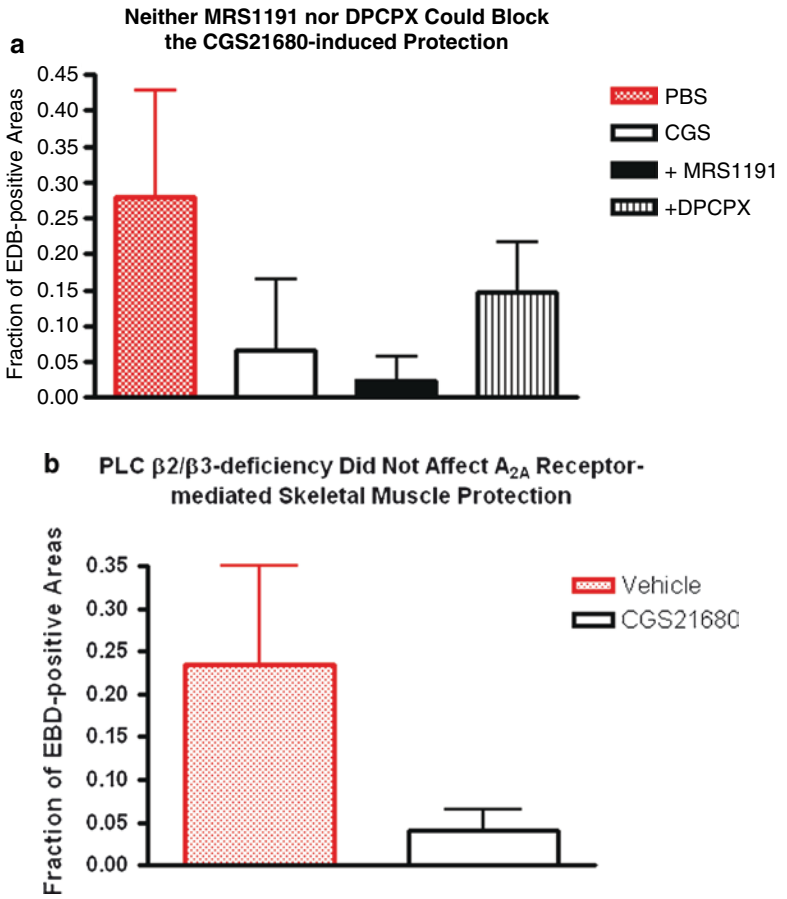


Fig. 13.3 Adenosine A_{2A} receptor agonist CGS21680-induced cytoprotection is not mediated via PLCβ2/β3. (**a**) Adult WT mice were injected with PBS vehicle, CGS21680, CGS21680 plus MRS1191 or CGS21680 plus DPCPX using method described in legend to Fig. 13.1. Following I/R, CGS21680 (nine mice) treatment caused a smaller extent of skeletal muscle injury as compared to PBS-(seven mice) treatment (one-way ANOVA and posttest comparison, $P < 0.05$). The CGS21680-induced decrease in I/R injury was not blocked by either DPCPX (ten mice) or MRS1191 (eight mice) ($P > 0.05$, CGS21680 vs. CGS21680 plus DPCPX or plus MRS1191) (**b**) PLCβ2/β3-null mice were pretreated with PBS vehicle (nine mice) or CGS21680 (six mice) and I/R injury determined. CGS21680 could still cause a significantly smaller extent of I/R injury than the vehicle PBS ($P < 0.05$, t-test). Data were expressed as mean ±SD. CGS21680: 0.07 mg/kg; SCH 442416: 0.017 mg/kg

13.3 Mechanism of Protection and Working Model of A_3 Receptor Signaling

Little is known regarding the effector mechanism of adenosine receptors in their protection against ischemia–reperfusion injury. In the pig latissimus dorsi muscle flap model, either glibenclamide or 5-hydroxydecanoate was able to reverse the

beneficial effect of ischemic preconditioning or that achieved by pre-ischemic intra-arterial infusion of adenosine. It was of interest to note that the protective effect was manifested as a reduction in the muscle infarct size, a higher muscle content of phosphocreatine, ATP and energy charge potential, and a lower lactate and muscle myeloperoxidase level.

In the murine hindlimb ischemia–reperfusion model, genetic absence of phospholipase C β2/β3 or PLCβ2/β3 (such as in PLCβ2/β3 null mice), abrogated the Cl-IBMECA-induced protection from ischemia–reperfusion injury in the skeletal muscle (Fig. 13.4). To rule out a nonspecific effect associated with Cl-IBMECA as the reason for its lack of cytoprotective effect in PLCβ2/β3 knockout animals, the protection mediated by a structurally different adenosine A_3 receptor-selective agonist, MRS3558 (Tchilibon et al. 2005), was investigated. MRS3558 also caused a large reduction in the EBD-positive skeletal muscle cells (Fig. 13.4). This protection was completely lost in the PLC β2/β3-null mice (Fig. 13.4). On the other hand, the protective effects of A_1 or A_{2A} receptors were unaffected in PLCβ2/β3 knockout mice. These data showed that the A_3 receptor signals selectively via the beta isozyme of PLC to exert its skeletal muscle protective effect and provided further evidence for a distinct cytoprotective property of each adenosine receptor.

The signaling cascade and the role of adenosine A_3 receptors on skeletal muscle cells, vasculature, and immune cells in mediating the beneficial effect of an A_3 receptor agonist in skeletal muscle are not known. In the working model illustrated in Fig. 13.5, activation of the A_3 receptor on skeletal muscle cells is linked to PLCβ2/β3, which in turn activates protein kinase C (PKC) via the PLC product diacylglycerol. PKC was shown to activate K_{ATP} channels and cause protection. That adenosine and ischemic preconditioning of the skeletal muscle have an energy-sparing effect is consistent with a consequence of sarcolemmal K_{ATP} channel activation. The latter can cause an abbreviation of the muscle action potential duration, reduced Ca^{2+} influx and overload, and thus ameliorated ischemic injury. The A_3 receptor protects the murine myocardium from ischemia–reperfusion injury by activating the sarcolemmal K_{ATP} channel (Wan et al. 2008). This raises the possibility for a potentially important role of the sarcolemmal K_{ATP} channel in skeletal muscle protection. Since Gq-coupled angiotensin II receptors can induce formation of reactive oxygen species (ROS) via the activation of NADPH oxidase (Griendling et al. 2000) and Gq is linked to activation of PLCβ, it is possible that the adenosine A_3 receptor is also coupled to ROS formation. ROS has been shown to cause a low-conductance opening of the mitochondrial permeability transition pore (mPTP), which may mediate the protective effect of ischemic preconditioning.

Another mechanism of A_3 protection is its anti-inflammatory effect, exerted not at the skeletal muscle level, but at the immune cells. In this signaling pathway, activation of the A_3 receptor on circulating immune cells will suppress their function and decrease immune cell-mediated damage to the skeletal muscle. In support of this concept is the finding that activated mast cells and neutrophils are important contributors if not mediators of skeletal muscle ischemia–reperfusion damage (Bortolotto et al. 2004; Formigli et al. 1992). Both mechanisms, involving activating the A_3 receptor on skeletal muscle cells and circulating immune cells, are not mutually exclusive. In fact, both can work in concert to reduce skeletal muscle

injury. Yet another signaling pathway which can afford protection is a potential A_3 receptor-mediated attenuation of injury that occurs during reperfusion following prolonged ischemia, or reperfusion injury (Jordan et al. 1999). Since reperfusion injury likely exists in the heart (Gross and Auchampach 2007), it is also quite possible that reperfusion injury occurs after ischemia in skeletal muscle. Other G protein-coupled receptors, such as the opioid receptor, have been linked to

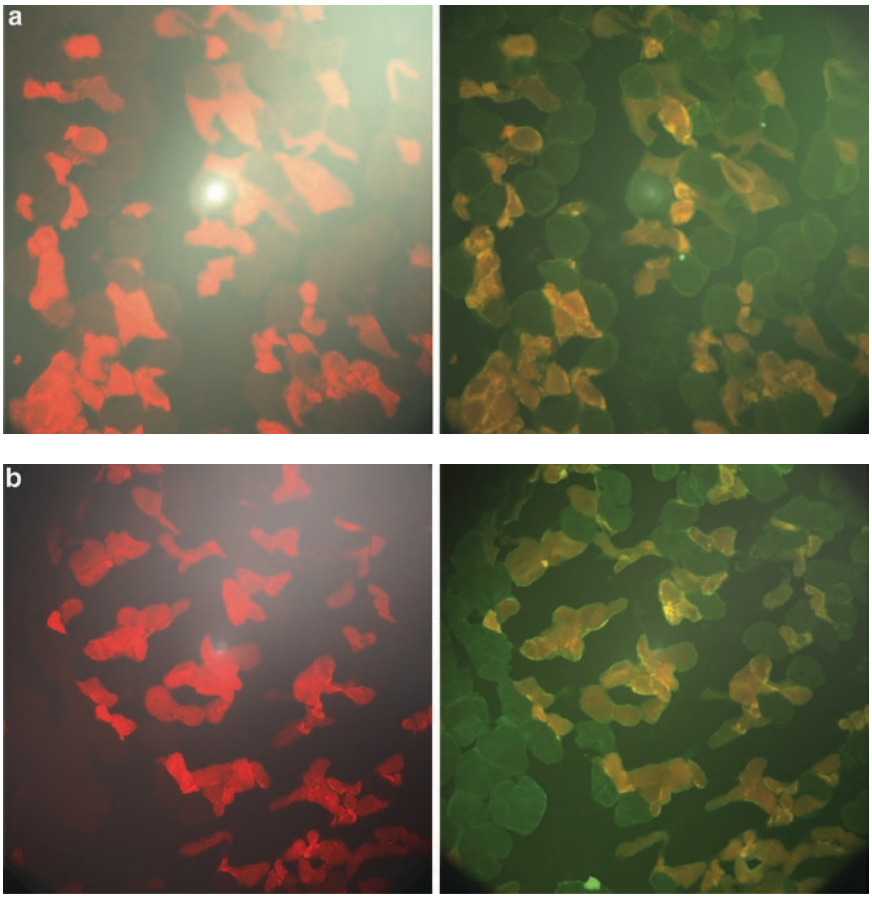


Fig. 13.4 The adenosine A_3 receptor signals through PLCβ2/β3 to cause its anti-ischemic skeletal muscle protective response. Adult PLCβ2/β3 null-mice were injected with vehicle (n = 9 mice), Cl-IBMECA (eight mice), MRS3558 (nine mice) or CCPA (ten mice), subjected to ischemia and reperfusion, and the extent of skeletal muscle injury was subsequently quantified as in WT mice. PLCβ2/β3 null-mice not subjected to ischemia/reperfusion of skeletal muscle obtained from the contra-lateral limb not subjected to ischemia/reperfusion did not show any EBD staining. (**a**) Vehicle-injected PLCβ2/β3 null-mice showed similar extent of EBD staining as did vehicle-injected WT mice (vehicle-injected WT not shown). (**b**) Cl-IBMECA did not reduce the % EBD-stained skeletal muscle in PLCβ2/β3 null-mice while

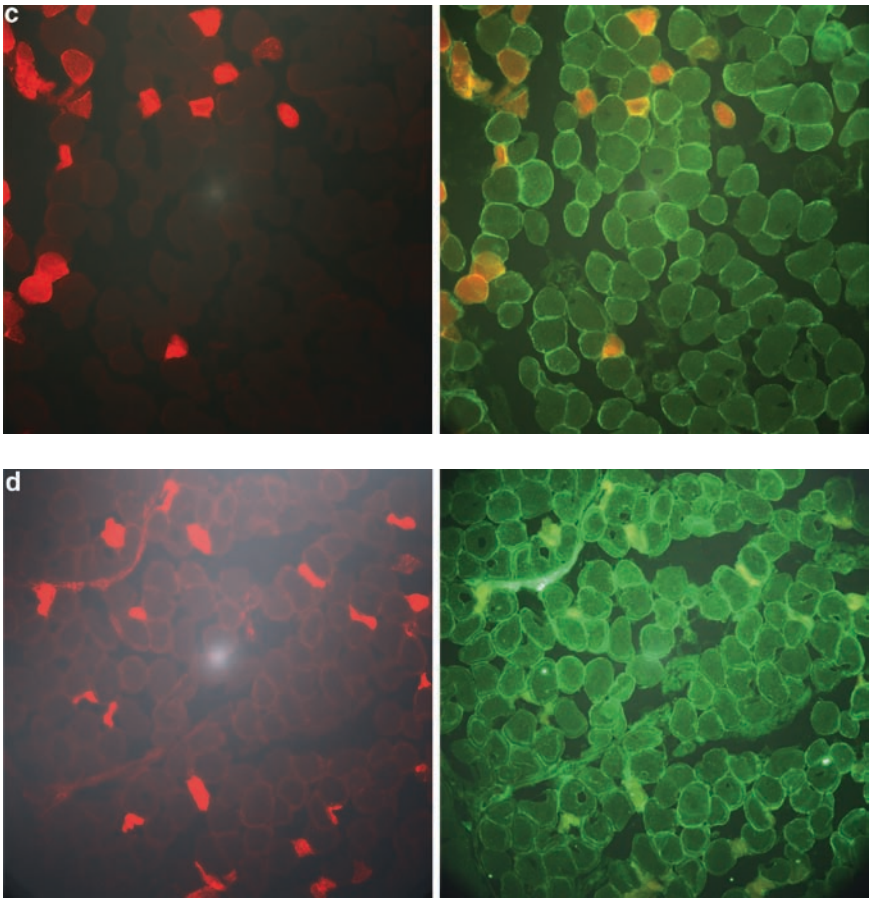


Fig. 13.4 (continued) (**c**) CCPA was effective in preventing the ischemia/reperfusion-induced injury in these mice. (**d**) A structurally distinct A_3 receptor agonist MRS3558 was also able to reduce skeletal muscle injury in WT mice (representative of eight mice)

activation of the PI-3 kinase, Akt and MAPK p42/p44 (Gross et al. 2004). These latter enzymes are known as reperfusion injury salvage kinase (RISK).

Concomitant expression of metallothioneins (MTs) and metalloproteinases (MMPs) occurs in skeletal muscle that has experienced an injury (Lecker et al. 2004; Warren et al. 2007). MTs are small (12–14 kDa), ubiquitous, cysteine-rich, zinc-binding proteins which are primarily produced in the liver and released into the circulation (Tapiero and Tew 2003). Upon release into the circulation these proteins play a pivotal role in cellular processes to render protection to all tissues of the body. In skeletal muscle, MTs initiate anti-inflammatory and anti-apoptotic signaling cascades, reduce reactive oxygen species (ROS)-induced cytotoxicity, protect against ROS-induced DNA degradation, and maintain zinc homeostasis

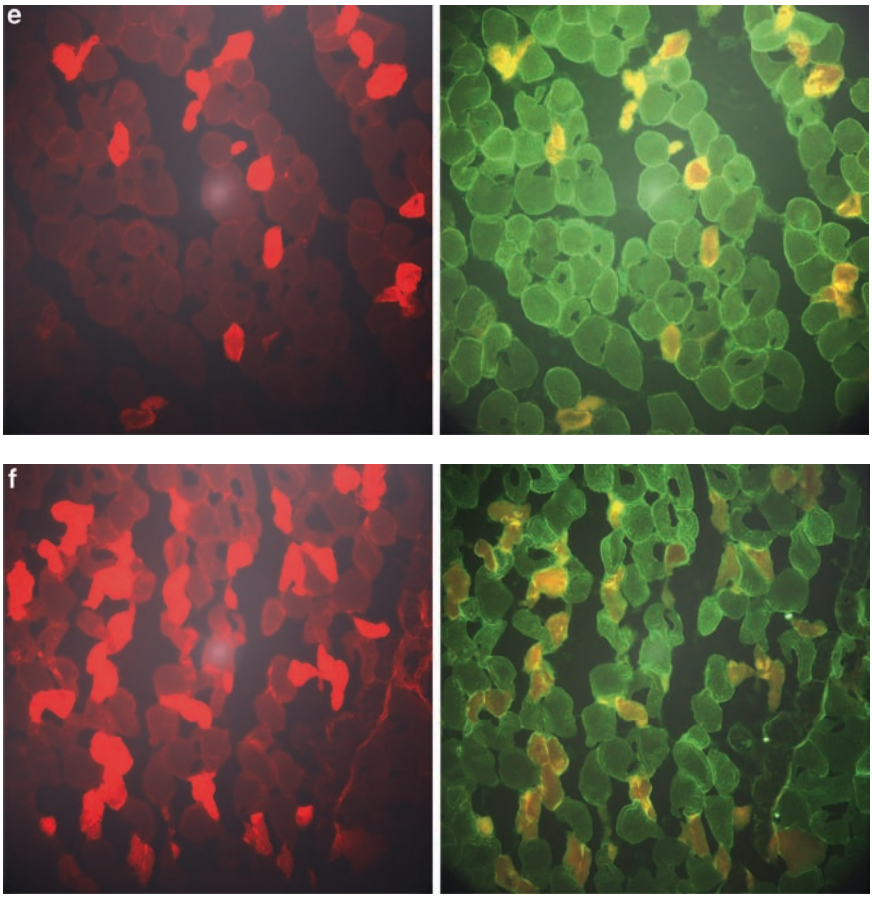


Fig. 13.4 (continued) (**e**) This reduction persisted in the presence of the A_1 receptor antagonist DPCPX in WT animals (n = 10 mice), in support of the concept that the protective response to this novel agonist is mediated by the A_3 but not the A_1 receptor. (**f**) The protective effect of MRS3558 was completely abrogated in PLCβ2/β3 null-mice, providing further evidence for the hypothesis that the A_3 receptor signals via PLCβ to achieve its cytoprotective effect

(Feng et al. 2006; Tapiero and Tew 2003; Miles et al. 2000). Marked induction of MT mRNA is evident in skeletal muscle of animals and humans under conditions that promote decreased protein synthesis and increased protein degradation, such as, muscle injury and disuse (Jagoe et al. 2002; Lecker et al. 2004; Kondo et al. 1994; Penkowa et al. 2005).

While the specific role of MTs is to neutralize ROS, MMPs contribute to tissue remodeling in both healthy and pathological muscle. MMPs process extracellular matrix proteins, cytokines and growth factors. Optimal remodeling of the extracellular matrix is contingent on tightly regulated MMP activity (Kjaer 2004). MMP

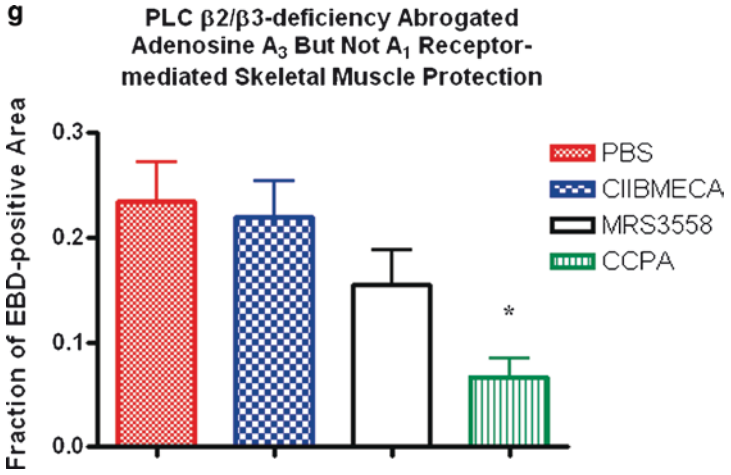


Fig. 13.4 (continued) (**g**) Average EBD staining (±SD) of skeletal muscle sections from PLCβ2/β3 null-mice following treatment with vehicle or adenosine ligand were quantified by blinded observers. *P < 0.05, CCPA vs. PBS, Cl-IBMECA or MRS3558

activity is largely dependent on the substrate affected, as specific MMPs are activated to degrade collagens (MMP-1, -8, -13, and -18), gelatins (MMP-2, and -9), stromelysins (MMP-3, -10, and -11), and membrane-type proteins (MMP-14, -15, -16, and -17).

Recent work has shown a concomitant increase in MT and MMP mRNA expression during skeletal muscle injury and subsequent remodeling (Jagoe et al. 2002; Urso et al. 2006; Warren et al. 2007). Several lines of evidence suggest an involvement of ROS in the cascade of events initiating skeletal muscle remodeling, particularly following injury when skeletal muscle cells are more susceptible to oxidative stress (Jagoe et al. 2002; Lecker et al. 2004; Warren et al. 2007). These findings imply that the signaling cascade connecting injury, the release of ROS, MT induction, and MMP-induced remodeling is a prime candidate for pharmacological intervention.

Pharmacological attenuation of MMP induction and downstream proteolytic cascades, or stimulation of MMP inhibitors such as TIMP-1 and TIMP-2, has the potential to prevent additional injury, while reducing the time to recovery (Hnia et al. 2007). Moreover, pharmacological agents designed to mitigate ROS-induced membrane damage by enhancing antioxidant molecules such as MT, may minimize the upregulation of proteolytic cascades in response to injury in skeletal muscle (Ghoshal et al. 1998; Nath et al. 2000).

The A_3 receptor agonist is a prime candidate for pharmacological intervention in the first few hours post-injury based on several working hypotheses regarding its mechanism of protection. First, the data in Fig. 13.4 in the previous section suggest that the activation of A_3 receptors is capable of inducing potent anti-ischemic

protection of the skeletal muscle and may mediate the protective effect of ischemic preconditioning. Potentially, A_3 receptor activation can induce a greater induction of MT following injury, providing the muscle with a greater antioxidant defense system subsequently suppressing oxidant-induced proteolytic cascades. A second working hypothesis regarding the mechanism of protection by the A_3 receptor agonist involves its anti-inflammatory properties in immune cells. The primary event linking skeletal muscle injury to intracellular proteolytic processes is the infiltration of inflammatory cells in the hours and days post-injury. It has been suggested that this inflammatory reaction may produce additional damage, increasing the possibility for muscle fibrosis, scarring, and subsequent injury (Tidball 1995).Thus, limiting certain aspects of inflammation through A_3 receptor modulation may reduce muscle degeneration as well as signaling mechanisms for muscle scarring (Sicard 2002). Finally, the role of the A_3 receptor agonist in mitigating Ca^{2+} influx and overload, through its effect on PKC signaling, has the potential to decrease the activation of MMPs and subsequent upregulation of downstream proteolytic cascades. Essentially, the mechanism for this protective response involves the disturbances in Ca^{2+} homeostasis resulting in elevated intracellular Ca^{2+}. This increase in cellular Ca^{2+} activates the cysteine protease calpain which plays a critical role in triggering skeletal muscle protein breakdown, inflammatory changes, and regenerative processes (Inserte et al. 2006; Stracher 1999; Mansoor et al. 1996). Figure 13.5 illustrates theproposed intracellular mechanisms involved in mitigating skeletal muscle injury following A_3 receptor agonist treatment. Based on these working hypotheses, we investigated the effects of pre-treatment with the A_3 receptor agonist Cl-IBMECA in skeletal muscle of mice following 2 h of ischemia in order to characterize the effect of A_3 receptor agonist treatment on transcription and translation of MT, MMPs, and TIMPs, at 24 h post-reperfusion injury. We compared the fold change of MT, MMP and TIMP mRNA expression in the injured versus the uninjured leg in PBS- and A_3agonist-treated mice.

Following ischemia–reperfusion injury, MT mRNA expression in the PBS treated animals was reduced 2.3-fold, although treatment with A_3 receptor agonist promoted a 26-fold increase in MT mRNA. Interestingly, despite this robust difference in mRNA, while protein levels of MT increased approximately 87% in the injured leg, there were no differences in MT protein levels between PBS- and A_3-receptor agonist treated animals. This robust increase in mRNA was not accompanied by any change in the protein level among treated vs. untreated animals. These results are likely due to the time point of sampling, such that alterations in levels of proteins may not be affected until approximately 48 h post-injury.

Regardless of the lack of evidence at the translational level, our data show that transcription of MMPs is significantly inhibited by A_3 receptor agonist treatment. MMP-2 expression increased 18.4-fold in the PBS treated animals, while A_3 receptor activation blunted this increase resulting in a modest 4.5-fold increase in MMP-2 mRNA. Similar results were seen for MMP-3 and MMP-14 with mRNA levels increasing 11.9- and 51.8-fold, respectively in the injured versus uninjured leg in PBS-treated animals. With A_3 receptor agonist intervention, however, MMP-3 and MMP-14 mRNA levels were 1.8-and 16.0-fold higher, respectively in the injured

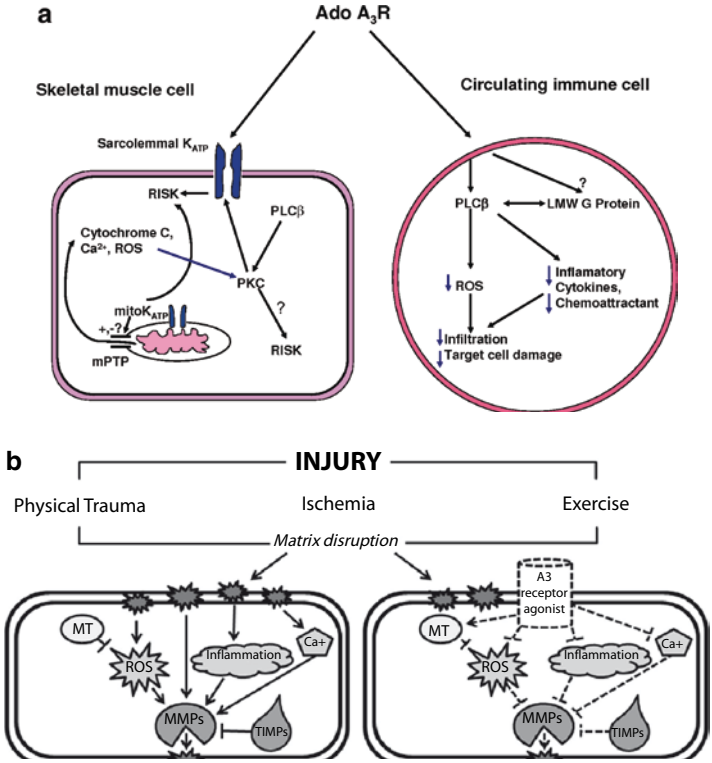


Fig. 13.5 Working models of adenosine A_3 receptor signaling mechanism. (**a**) Models of adenosine A_3 receptor signaling mechanism in cytoprotection of skeletal muscles. The cytoprotective effect of adenosine A_3 receptors can be achieved at the levels of both circulating immune cells and skeletal muscle cells. PLCβ may mediate effects in both types of cells (Zheng et al. 2007). In immune cells, an anti-inflammatory effect is important. In skeletal muscle cells, PKC and RISK are key enzymes that decrease injury. mitoK$_{ATP}$ can modulate mPTP and subsequent release of the pro-apoptotic cytochome C, ROS and calcium. Inhibition of mPTP reduces injury. Other mechanism such as modulation of MMPs, TIMPs, and MT is also potentially important (see Fig. 13.5b). PKC, protein kinase C; mitoK$_{ATP}$, K$_{ATP}$ channels in mitochondria; RISK, reperfusion injury salvage kinases; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species. (**b**) Working model of the relationship between skeletal muscle injury, intracellular signaling, and potential models of A_3 receptor activation on these pathways. The left panel depicts the natural pathology associated with skeletal muscle injury (*solid lines*). The right panel illustrates the proposed signaling pathways affected by A_3 receptor agonist treatment (*dashed lines*). Arrows represent actions that promote the activity of downstream molecules, while *flat lines* indicate mitigation or inhibition. *Solid lines* represent normal and pathological processes in skeletal muscle. MMP-matrix metalloproteinase, MT-metallothionein, ROS-reactive oxygen species, TIMP-tissue inhibitor of metalloproteinase

versus the uninjured leg. Interestingly, MMP-9 mRNA was decreased 1.9-fold in the animals receiving the vehicle and upregulated 5.6-fold in the A_3 agonist treated animals. Due to the role of MMP-9 as an anti-inflammatory agent, these results are encouraging. As far as the inhibitors of the metalloproteinases, TIMP-1 and TIMP-2,

mRNA data indicate that pre-treatment with the A_3 receptor agonist promotes enhanced MMP inhibition, with TIMP-1 mRNA expression increasing 9.1-fold in the A_3 agonist treated animals and only 3.9-fold in the vehicle treated animals. There were no significant differences in TIMP-2 expression among the treatment groups, with TIMP-2 expression levels increasing approximately twofold in the injured leg.

13.4 Future Directions

Future investigations will be fruitful if genetic and receptor pharmacological tools can be combined to more definitively answer both the function and signaling mechanism subserved by each adenosine receptor subtype. For example, gene knockout mice deficient in an adenosine receptor subtype can help delineate the role played by circulating immune cells, for example, by studying whether bone marrow transplant from A_3 adenosine receptor-null mice will either show an attenuated protection or fail to demonstrate any protection by an A_3 agonist. Similarly, the role of sarcolemmal vs. mitochondrial K_{ATP} channels in mediating the protective effect of A_3 receptors is an important question to answer. The use of sarcolemmal- and mitochondrial-selective K_{ATP} channel blockers and openers will be useful, as will testing whether A_3 protection of skeletal muscle is lost in Kir6.2 KO mice lacking the pore-forming subunit of the sarcolemmal K_{ATP} channel. Another interesting question is whether PLC beta is required for K_{ATP} channel activation if the latter is an effector of the adenosine A_3 receptor. The role of A_3 receptors in modulating MT, MMPs and TIMPs is interesting and potentially important. mRNA alterations are truly robust and indicate that A_3 receptor agonist treatment provides protection at the transcriptional level. Indeed, the lack of protein data to support preliminary findings limits the absolute certainty that A_3 agonist treatment will provide protective benefits via the MT pathway in skeletal muscle following injury. However, it is likely, based on skeletal muscle translational efficiency, that alteration in the protein level of MTs, MMPs, and TIMPs follows a slower time course. Overall, these preliminary data suggest that activation of the adenosine A_3 receptor may modulate MTs, MMPs and TIMPs in skeletal muscle.

13.5 Disclaimer

The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. In conducting the research described in this report, the investigators adhered to the “Guide for Care and Use of Laboratory Animals” as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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Chapter 14
A_3 Adenosine Receptors, HIF-1 Modulation and Atherosclerosis

Stefania Gessi, Stephen MacLennan, Edward Leung, and Pier Andrea Borea

14.1 Introduction

Atherosclerosis, a multifactorial disease of the large arteries, is the major cause of heart disease and stroke worldwide. Epidemiological studies have discovered several relevant environmental and genetic risk factors associated with this pathology (Lusis 2000). Genomic and proteomic-based strategies in humans and rodent models have been instrumental in discovering genes and proteins involved in the initiation and progression of atherosclerosis. Moreover multiple genome-wide approaches are underway to identify variants in the human genome that predispose to, or protect from, cardiovascular disease (Adhikari et al. 2006). Atherosclerosis has traditionally been viewed to simply reflect the deposition of lipids within the vessel wall of the large arteries. This concept has changed (Stoll and Bendszus 2006). It is now accepted that a complex endothelial dysfunction induced by elevated and modified levels of low-density lipoproteins (LDL), free radicals, infectious microorganisms, shear stress, hypertension or combinations of these and other factors leads to a compensatory inflammatory response (Ross 1999). Endothelial dysfunction is characterized by decreased nitric oxide synthesis, local oxidation of circulating LDL and their entry into the vessel wall. The early lesions of atherosclerosis consist of subendothelial accumulations of cholesterol-engorged macrophages, called "foam cell". Foam cells are characteristic pathological cells in the lesions of atherosclerosis. During the process of atherosclerosis, monocytes seem to play a

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central role. Once monocytes adhere to the subendothelial space and enter into the intima of the artery, oxidatively modified LDL (oxLDL) may participate in activation of the monocytes into macrophage. Uptake of oxLDL by the macrophages through scavenger receptors will lead to foam cells formation (Libby 2002). Cholesterol accumulation in macrophages not only contributes to cholesterol retention within the vessel wall, but also alters macrophage biology (Sluimer et al. 2008). Indeed a number of cytokines and enzymes known to be induced by hypoxia are expressed by foam cells in human atherosclerotic plaques including VEGF, PDGF, TNFalpha, IL-1, MMP1, MMP7.

Our views of the pathophysiology of atherosclerosis have evolved substantively over the past century and now it is recognized that inflammation and angiogenesis are important features in atherosclerotic plaque development and destabilization (Vink et al. 2007; Kolodgie et al. 2007). The anoxemia theory of atherosclerosis highlights the fact that an imbalance between the demand and supply of oxygen in the arterial wall is a key factor for the development of atherosclerotic lesions. When atherosclerotic lesions develop, the arterial wall thickness increases and diffusion capacity is impaired. At the same time, oxygen consumption is augmented, and an energy imbalance may occur (Björnheden et al. 1999). Recent studies have documented increased neovascularization in complex atherothrombosis (Moreno et al. 2006). Intraplaque neoangiogenesis may contribute to atherogenesis by providing a vascular network for inflammatory cell infiltration (Herrmann et al. 2006). In addition, the presence of microvessels is associated with intraplaque hemorrhage, which leads to rapid plaque progression (Vink et al. 2007). The clinical complications of atherosclerosis are caused by thrombus formation, which in turn results from rupture of an unstable atherosclerotic plaque.

14.2 HIF-1 in the Pathogenesis of Atherosclerosis

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that is regarded as the major oxygen homeostasis regulator (Semenza 2001). HIF-1 is only active as a heterodimer that is composed of two subunits: HIF-1α and HIF-1β. HIF-1β is constitutively expressed, whereas the expression of HIF-1α is regulated. In normal oxygen conditions, HIF-1α is translated and rapidly degraded by the proteosome. During hypoxia, the degradation of HIF-1α is blocked with subsequent rapid increase of HIF-1α levels. This permit dimerization with the HIF-1β subunit to form the active HIF-1 complex in the nucleus. The heterodimer HIF-1 binds to the hypoxic response elements of target gene regulatory sequences, resulting in the transcription of genes implicated in angiogenesis, cellular metabolism and apoptosis. The presence of plaque hypoxia is primarily determined by plaque inflammation (increasing oxygen demand), while the contribution of plaque thickness (reducing oxygen supply) seems to be minor. Inflammation and hypoxia are almost interchangeable and both stimuli may initiate HIF-driven angiogenesis in atherosclerosis

(Sluimer and Daemen 2009). Angiogenesis describes the formation of new capillaries from post-capillary venules, typically stimulated by tissue ischemia/hypoxia via action of HIF-1. Promoter regions for the stabilized HIF-1 dimer belong to gene sequences encoding, for instance, for VEGF, which stimulates endothelial cell proliferation, migration, survival and mediates vascular hyperpermeability. By this latter action, VEGF facilitates a fibrin network for the proliferating endothelial cells once proteolytic degradation of the basement membrane via action of matrix metalloproteinases MMP2 and MMP9 allows their sprouting into the extracellular matrix. Increased expression of HIF, VEGF, MMP2, MMP9 in the coronary artery wall was shown to coincide with vasa vasorum neovascularization. Neovessels within the atherosclerotic plaque are characterized by paucity of tight junctions, a discontinuous basement membrane and a relative lack of smooth muscle cells. Thereby these neovessels are not only leaky but also unable to control intraluminal pressure and therefore prone to rupture.

VEGF expression in atherosclerotic plaque has been described in connection with plaque angiogenesis and progression (Inoue et al. 1998; Zhao et al. 2002). Preclinical studies showed that the formation and progression of atherosclerotic plaques are attributed to the VEGF-mediated increase of angiogenesis and the recruitment and activation of monocytes (Paul et al. 2004). Furthermore, it has been demonstrated, using conditional knockouts of HIF-1α, that HIF-1α plays an essential role in the direct regulation of survival and function in the inflammatory response (Cramer et al. 2003). Recently it has been demonstrated the presence of HIF-1α in atherosclerotic lesions and its association with an atheromatous inflammatory plaque phenotype and with VEGF expression (Vink et al. 2007). The role of HIF in the formation and progression of human coronary atherosclerosis has to be further investigated and the downregulation of HIF-1, as a strong inducer of VEGF, must be considered as a possible target of preventing plaque formation (Paul et al. 2004).

Recently it has been reported that ox-LDL provoked HIF-1α accumulation in human macrophages (Shatrov et al. 2003). HIF-1α accumulations triggered by ox-LDL have also been detected in patients with atherosclerosis, but the role of these accumulations in the pathophysiology of atherosclerosis has not been determined. Macrophages are central for both inflammation and lipid deposition during atherogenesis. It has been demonstrated that HIF-1α-siRNAs blocks the development of macrophage derived foam cells by inhibiting expression of HIF-1α (Jiang et al. 2007). This strongly suggests that under atherogenic conditions, the high expression of macrophage HIF-1α promotes foam cell formation and atherosclerosis. Furthermore it has been demonstrated that HIF-1α-siRNAs inhibit the increase of the expression of the majority of atherosclerosis-related genes induced by ox-LDL. Recent advances in basic science have established a fundamental role for inflammation in mediating all stages of this disease from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis. The general increase in IL-1β, COX-2, ICAM-1 and VCAM-1 induced by ox-LDL, confirms the concept of inflammation in atherosclerosis (Libby 2002).

Therefore the induction of HIF-1α by atherogenic factors may be a key step in coordinating the cellular events that result in atherosclerotic lesions by linking inflammation and atherosclerosis through the mechanism of induction the expression of the majority of atherosclerosis-related genes. It is noteworthy that recently a series of molecules and molecular pathways emerged that contribute to the pathogenesis and progression of both atherosclerosis and cancer (Ross et al. 1999; Toi et al. 2002). HIF-1 may be added to that list, considering the important tumor angiogenic role of HIF-1α.

14.3 Adenosine Receptors and HIF-1

Hypoxia markedly changes cellular metabolism and causes the accumulation of extracellular adenosine (Van Belle et al. 1987; Blay et al. 1997). This accumulation is at least partially explained by hypoxia-mediated regulation of enzymes that are involved in adenosine metabolism, adenosine kinase and 5′-nucleotidase (Decking et al. 1997; Kobayashi et al. 2000; Thompson et al. 2004; Synnestvedt et al. 2002). Different studies demonstrated a link between ado and HIF-1 at first in human cancer cell lines and then also in murine macrophages and in liver cells (Merighi et al. 2005; 2006; 2007; Ramanathan et al. 2007; de Ponti et al. 2007; Clark et al. 2007; Alchera et al. 2008; Wendler et al. 2007). In most of these cases its accumulation was related to an increase of the angiogenic factor VEGF, which regulates important functions associated with angiogenesis. Different receptor subtypes have been reported to play a role in the adenosine-induced HIF-1α accumulation depending on the cellular model investigated. In particular in human cancer cell lines it has been demonstrated that through transcriptional and translational mechanisms the A_3 receptor was responsible for HIF-1 accumulation under hypoxic conditions. The HIF-1 regulation by A_3 activation was dependent on pERK1/2, p38 and pAkt activation in carcinoma cells, and induced an increase in angipoietin-2 or VEGF depending on the cell model investigated (Merighi et al. 2005, 2006, 2007). In murine macrophages as well as in liver cells it has been reported that the A_{2A} receptor was involved in HIF-1α accumulation in normoxic conditions (De Ponti et al. 2007). Contrasting results reported that A_{2A} receptor activation in murine macrophages induced HIF-1 only after stimulation with LPS by increasing mRNA stability (Ramanathan et al. 2007), whilst other data revealed that A_{2A} stimulation alone increased HIF-1 levels by activating the pAkt/mTOR pathway (Ramanathan et al. 2007; De Ponti et al. 2007; Alchera et al. 2008). As for hypoxic embryos it was found that knock out animals for the A_1 subtype had less stabilized HIF-1α and much more severe growth retardation than wild type embryos, suggesting that short periods of hypoxia during early embryogenesis can result in intrauterine growth retardation and that the A_1 adenosine receptor played an essential role in protecting the embryo from hypoxia (Wendler et al. 2007).

14.4 A_3 Adenosine Receptors, HIF-1 and Atherosclerosis

A role for A_3 receptors in modulation of atherogenesis has been explored in double knock out mice for apolipoprotein E and A_3 receptors, following an high fat diet inducing atherosclerosis development (Jones et al. 2004). After aorta isolation it has been demonstrated that the lack of A_3 subtype induced a reduction in vascular smooth muscle cell proliferation in vitro and increased the expression of matrix enzyme lysyl oxidase, an enzyme involved in collagen and elastin cross-linking, that is essential in maintaining endothelial extracellular matrix (ECM) structure. However A_3-deficiency did not attenuate the development of atherosclerotic lesions in response to high fat diet or vascular injury in vivo suggesting that A_3 receptors were not essential for vascular plaque formation. However developmental compensatory mechanisms occurring in response to A_3 receptors genetic ablation may be partially responsible for the discrepancy between in vitro and in vivo studies.

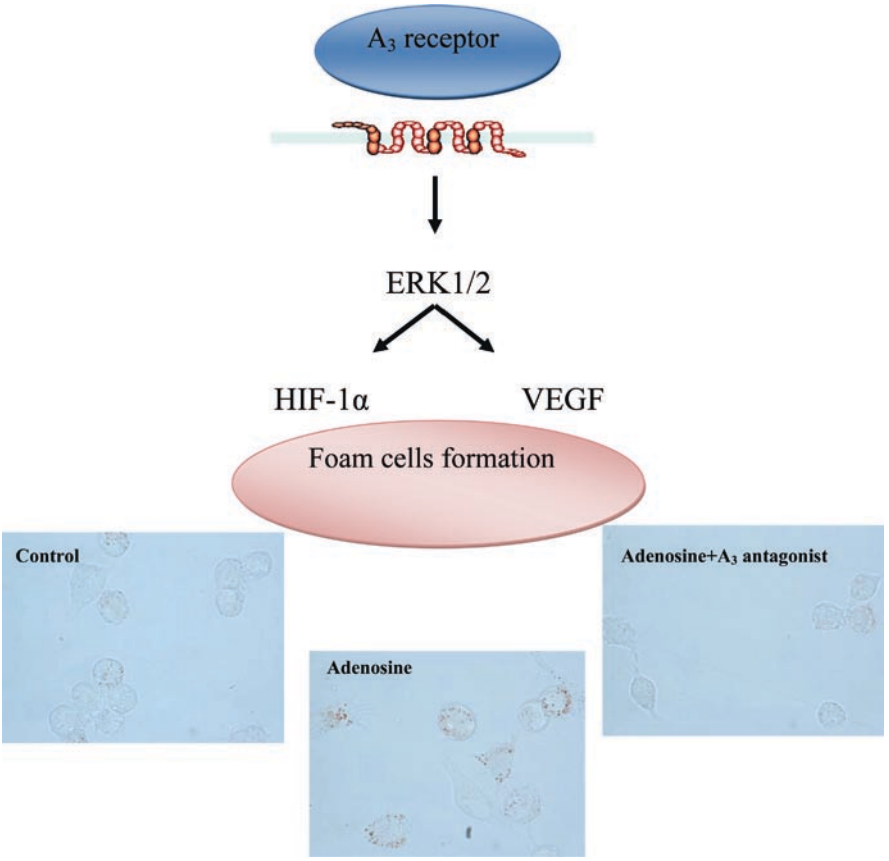

Fig. 14.1 Foam cells obtained after addition of oxLDL, incubated with adenosine in the absence and in the presence of A_3 adenosine receptor antagonist (Oil red O staining)

Furthermore it is also relevant underline that in the case of A_3 receptors due to the species difference of the receptor the murine model may have not been suitable to study the real contribution of this adenosine subtype to atherosclerosis and data from mice should be interpreted with caution. It has been clearly demonstrated that both agonists and antagonists present different binding affinities for the murine and human A_3 subtype and the reported paradoxical protection in A_3 knock-out hearts despite A_3-mediated protection in wild-type hearts, could indicate limitations of gene-knock-out models (Jacobson and Gao 2006). Recently by using an in vitro approach it has been demonstrated that adenosine was able to induce HIF-1α accumulation under hypoxic conditions in human macrophages and in a cellular model of human foam cells through activation of different adenosine receptors including the A_3 subtype (manuscript submitted). The A_3 selective agonist Cl-IB-MECA was able to potently increase HIF-1 accumulation in macrophages and foam cells with an EC_{50} in the low nanomolar range and the A_3 antagonist MRE 3008F20 potently reverted its effect. A_3 receptor-dependent modulation of HIF-1 required ERK 1/2 phosphorylation and then was responsible for an increase in VEGF and foam cells formation (Fig. 14.1).

14.5 Conclusions

Current therapeutic strategies to prevent atherosclerosis are primarily based on the use of statins, inhibitors of the novo cholesterol synthesis that decrease serum LDL cholesterol levels thereby inhibiting the uptake of native and oxidatively modified LDL by macrophages in the arterial wall (Fonarow and Watson 2003; Kastelein 2003). Despite the proven effectiveness of statins and their widespread use, the incidence of cardiovascular disease still remains high, indicating that there is an important need for new alternative therapies (Pennings et al. 2006). In view of the relevance of HIF-1 in atherosclerosis, following its effects in angiogenesis, inflammation and foam cells formation, the evidence around HIF-1 modulation by A_3 receptors activation strongly suggest a novel role for A_3 antagonists in the protection from atherosclerotic plaque formation.

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Part VI
Inflammatory and Auto-Immune Diseases

Chapter 15
Rheumatoid Arthritis: History, Molecular Mechanisms and Therapeutic Applications

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15.1 Rheumatoid Arthritis: Background

Rheumatoid arthritis (RA), a chronic autoimmune inflammatory disorder of unknown etiology that occurs in approximately 1% of the population (Albani and Carson 1997). In all populations, RA is more prevalent among women than men, and usually develops in the fourth and fifth decades of life, with 80% of the total cases occurring between the ages of 35 and 50 (Kavanaugh and Lipsky 1996). The primary presenting symptoms are pain, stiffness, and swelling of the joints resulting in impaired physical function. These symptoms are often accompanied by constitutional symptoms such as fever and malaise (Grassi et al. 1998). Synovial inflammation underlies the cardinal manifestations of this disease, which include pain, swelling, and tenderness followed by cartilage destruction, bone erosion, and subsequent joint deformities. In RA, joint involvement is typically symmetric, a characteristic usually not found in other forms of arthritis (Muller-Ladner et al. 2005; Majithia and Geraci 2007).

Despite intensive research, the precise cause of RA remains elusive. Although a variety of cells play a role in RA disease progression, macrophages may be of particular importance. Once in the synovium, macrophages are capable of antigen presentation and T-cell activation. Moreover, the extent of macrophage infiltration into the synovium correlates with RA severity and progression (Maruotti et al. 2007). Macrophage-derived cytokines, such as tumor necrosis factor alpha (TNF-α), appear to play a critically important role in the induction and perpetuation of the chronic inflammatory processes in rheumatoid joints as well as in the systemic manifestations of this disease (Grossman and Brahn 1997). TNF-α is a key inflammatory mediator. This cytokine is overproduced in joints of patients with RA and triggers increases in synoviocyte proliferation and a cascade of secondary mediators involved in the recruitment of inflammatory cells and in the process of joint destruction (Camussi and Lupia 1998). Joint erosion is known to occur early in RA, affecting about 40% of the patients during the first year and 90% during the

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first 2 years (Plant et al. 1998). Elevations in inflammatory markers are antecedents of disease progression and joint destruction in early RA (Matsuda et al. 1998). Indeed, the rate of cartilage and joint damage is correlated with plasma elevations in inflammatory acute phase reactants, such as C-reactive protein (CRP) and vascular endothelial growth factor, and in the synovial concentrations of matrix metalloproteinase's, matrix digesting enzymes directly responsible for joint destruction (Paleolog 2002; Burrage et al. 2006; Varghese 2006).

The treatment of RA has undergone somewhat of a revolution over the last decade, with a strong consensus emerging in favor of early, aggressive therapy (Lee and Weinblatt 2001; O'Dell 2001; Goldbach-Mansky and Lipsky 2003; Scott and Kingsley 2006). There is now evidence that early treatment of the disease has a beneficial impact on treatment outcome. The goals to be achieved in managing RA are prevention or control of joint damage, prevention of loss of function, and reduction of pain (Sizova 2008). Non-steroidal anti-inflammatory drugs (NSAIDS), glucocorticoid joint injections, and/or low-dose prednisone may be considered for control of symptoms (Kirwan 1995; Lim and Conn 2001; Kavanaugh 2007).

In light of the therapeutic paradigm shift to early and aggressive treatment, the majority of patients with newly diagnosed RA are started on disease-modifying antirheumatic drug (DMARD) therapy within 3 months of diagnosis. Methotrexate, a powerful immunosuppressive and anti-inflammatory agent, is probably the most commonly used DMARD and is one of the most consistently effective ones (Lee and Weinblatt 2001). Furthermore, the past 7 years have seen the introduction of seven new DMARDs which include leflunomide, the highly specific and efficacious anticytokine agents, including adalimumab, etanercept, and infliximab, and recently, abatacept, and rituximab, and others (Goldbach-Mansky and Lipsky 2003; Scott and Kingsley 2006). These therapies are emerging as important and successful therapeutics for patients with early disease (O'Dell 2001; Goldbach-Mansky and Lipsky 2003; Scott and Kingsley 2006). Although effective in many patients, they are not without their drawbacks. Methotrexate and Leflunomide, require careful monitoring and can cause serious hepatic and pulmonary toxicities (Lee and Weinblatt 2001). The anti-TNF-α biological agents are costly, require parenteral administration, and have been associated with serious and opportunistic infections and lymphoma (Lee and Weinblatt 2001; Goldbach-Mansky and Lipsky 2003; Scott and Kingsley 2006). Furthermore as there are no 'cures', patients will require 30–40 years of ongoing therapy; although most of these agents do not remain effective in an individual for longer than 5 years. Thus, despite major recent advances in the treatment of RA, there is still need for convenient, safe, and effective therapies for many patients.

15.2 A_3AR Agonists: Anti-inflammatory Agents for the Treatment of RA

The findings showing up-regulation of the A_3AR in inflammatory tissues of patients with RA prompted studies which led to the utilization of this receptor as a therapeutic target (Ochaion et al. 2006; Bar-Yehuda et al. 2007; Madi et al. 2007).

To explore the anti-inflammatory effect of synthetic A_3AR agonists and to look at the mechanism of action mediated down-stream to receptor activation, *in vitro* and *in vivo* studies were conducted.

The anti-inflammatory effect of the agonists was first proved in *in vitro* studies in fibroblast like synoviocytes (FLS) derived from synovial fluid of patients with RA. The cells were cultured and served as the first screening to study the effect of the agonists on inflammatory cells. The CF502 A_3AR agonist (generically known as MRS3558) possessing high affinity and selectivity at the human A_3AR, induced a linear dose dependent inhibitory effect on the proliferation of FLS via inhibition of TNF-α (Tchilibon et al. 2005; Ochaion et al. 2008).

Earlier work in experimental animal models, demonstrated that the A_3AR is highly expressed in the inflamed synovial tissue in comparison to low expression in naïve animals. Moreover, the high receptor expression was also reflected in the peripheral blood mononuclear cells (PBMCs) of the arthritic animals (Fishman et al. 2006; Ochaion et al. 2006, 2008; Rath-Wolfson et al. 2006; Bar-Yehuda et al. 2007). Upon oral treatment with the selective A_3AR agonists CF101 (generically known as IB-MECA) and CF502, disease was ameliorated and a marked decrease in disease clinical manifestations was recorded. CF101 treatment reduced inflammation, pannus formation, cartilage destruction and bone lyses (Baharav et al. 2005; Fishman et al. 2006; Ochaion et al. 2006, 2008; Rath-Wolfson et al. 2006; Bar-Yehuda et al. 2007). The specificity of the response was evidenced when an antagonist to the A_3AR was introduced to the arthritic animals prior to each agonist treatment, neutralizing the anti-inflammatory response (Fishman et al. 2006). A point to note is that the animals were treated twice or thrice daily via an oral route, approving former assumption that the molecule is stable and systemically absorbed via the intestine (Bar-Yehuda et al. 2007). Interestingly, shortly after treatment, A_3AR protein expression levels were down-regulated in the synovial tissue, lymph node and spleen of the AIA animals, demonstrating that receptor was internalized and degraded within the cells. Upon chronic treatment with CF101, receptor turned to its high levels again after 8–12 h (Fishman et al. 2006; Ochaion et al. 2006, 2008; Rath-Wolfson et al. 2006; Bar-Yehuda et al. 2007). It is important to note that receptor de-sensitization was transient and have not led to receptor tachyphylaxis, maintaining the A_3AR a valid target along the treatment period.

15.3 Anti-inflammatory Effect of A_3AR Agonists: Molecular Mechanism

The A_3AR is a 7 trans-membrane Gi-protein coupled inhibitory (Gi) receptor and its activation inhibits adenylyl cyclase activity and cAMP formation (Zhao et al. 2000). *In vitro* and *in vivo* studies show that treatment of inflammatory cells with A_3AR agonists induce modulation of signaling proteins, downstream to receptor activation, leading to de-regulation of the NF-κB signaling pathway and apoptosis of inflammatory cells. More specifically, in fibroblasts-like synoviocytes derived from RA patients as well as in synovial cells and in DLN derived from CF101

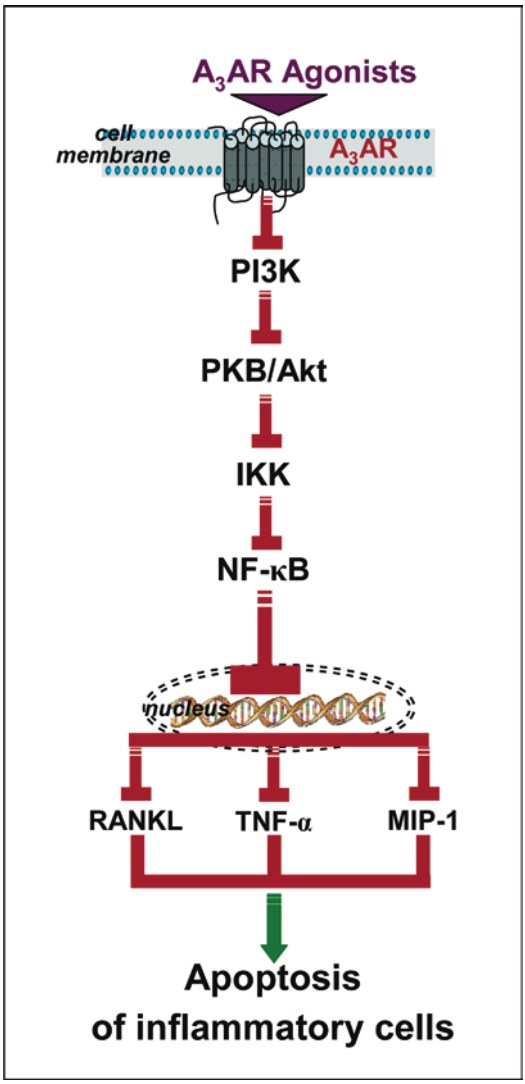
arthritic animals, a decrease in the expression levels of PI3K, phosphorylated PKB/Akt, IKKα/β, NF-κB and TNF-α protein was noted. In addition, RANKL was down-regulated as well, attributing to the ability of CF101 to prevent bone resorption (Baharav et al. 2005; Fishman et al. 2006; Ochaion et al. 2006, 2008; Rath-Wolfson et al. 2006; Bar-Yehuda et al. 2007). The expression level of the chemokine macrophage inflammatory protein 1 alpha (MIP-1α) was also decreased upon CF101 treatment (Szabó et al. 1998). Moreover, an increase in the expression level of caspase-3 took place, suggesting that apoptosis occurred upon CF101 treatment (Fishman et al. 2006; Rath-Wolfson et al. 2006; Bar-Yehuda et al. 2007). The extended lifespan of rheumatoid inflammatory cells such as neutrophils, lymphocytes, macrophages, fibroblasts and synoviocytes in the joints, and other inflammatory sites, is part of the pathogenesis of rheumatoid arthritis (Pap et al. 2000; Wang et al. 2003). One of the mechanisms that can contribute to this phenomenon is inhibition of apoptosis due to stimulation of the PI3K pathway, which leads to activation of PKB/Akt. The latter phosphorylates several proteins such as GSK-3β, FKHR and BAD, which then fail to induce apoptosis. It may also prevent the expression of caspase-9 and caspase-3, proteins pivotal in the apoptotic cascade. Over-expression and activation of PKB/Akt have been defined as the main barrier of apoptosis in the inflamed rheumatoid arthritis tissues (Stoica et al. 2003; Yang et al. 2003). PKB/Akt inhibition by CF101 and the increase in caspase-3 expression level in the CF101-treated animals, support the role of this pathway in ameliorating the inflammatory process (Scheme 15.1).

Beside the direct anti-inflammatory effect, CF101 was found to exert an immuno-modulatory effect in the AIA model. CF101 inhibited the proliferation of auto-reactive T cells derived from CF101-treated AIA rats (Bar-Yehuda et al. 2007). The drug also prevented the induction of arthritis in rats via adoptive transfer experiments. AIA can be transferred from one animal to the other upon engrafting spleen or lymph node cells from an arthritic animal to a naive one (Taurog et al. 1983; Spargo et al. 2006; Bar-Yehuda et al. 2007). Taken together, CF101 exerts its immuno-modulatory effect via inhibition of pro-inflammatory cytokine production and improvement of T cell function (Scheme 15.2).

15.4 The Clinical Development of CF101 as an Anti-inflammatory Drug to Combat RA

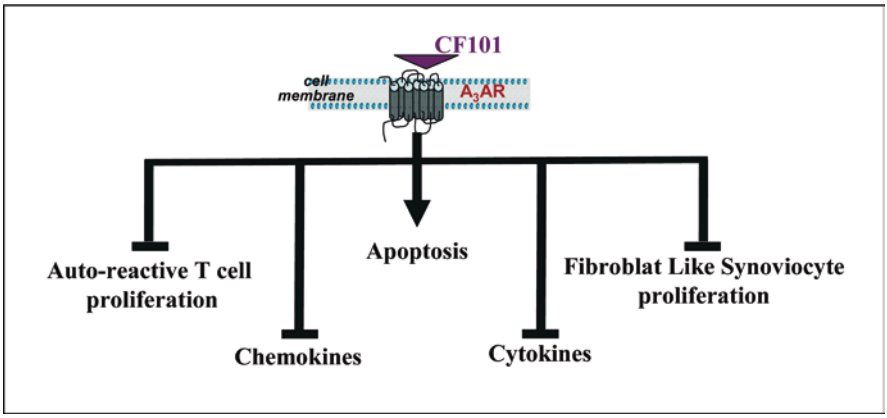
The excellent chemical and pharmacological profile of CF101 together with the findings showing up-regulation of the A_3AR in patients with RA, prompted the initiation of a clinical program aiming at the development of CF101 as a drug candidate for the treatment of RA.

Pre-clinical studies resulted in the development of a robust route of synthesis for CF101 in a large scale production. The active pharmaceutical ingredient was found to be highly stable for a long period of time. CF101 was slowly metabolized in mouse, rat, rabbit, monkey and human hepatocytes and studies with human hepatic



Scheme 15.1 Molecular mechanism involved with the anti-inflammatory effect of A_3AR agonists

microsomes indicated that no drug interactions, related to inhibition of the CYP450 metabolism, took place. Chronic toxicity studies in mice and monkeys revealed no cardiovascular risks and no toxicological issues (Bar-Yehuda et al. 2007). In a Phase I study in healthy subjects, CF101 was found to be safe and well tolerated with a linear pharmacokinetic activity (van Troostenburg et al. 2004). In a Phase IIa study conducted in patients with RA, CF101 administered twice daily for 12 weeks resulted in an improvement of disease signs and symptoms and appeared to be safe



Scheme 15.2 Immuno-modulatory effects mediated via the A_3AR

and well tolerated. Analysis of A_3AR expression levels at base line showed statistically significant direct correlation with patient response to CF101, suggesting A_3AR utilization as a biomarker to predict patients' response to the drug prior to treatment initiation (Silverman et al. 2008).

To conclude, the A_3AR which is highly expressed in inflammatory cells of AIA rats and patients with RA, is suggested as a biological marker and therapeutic target in RA. Synthetic agonists, which bind with high affinity and selectivity to the receptor, induce a marked anti-inflammatory effect mediated via de-regulation of the NF-κB signaling pathway, resulting in inhibition of pro-inflammatory cytokines and in apoptosis of inflammatory cells. The oral bioavailability of the A_3AR agonists and the clinical data produced in the Phase IIa human study support the development of these agents as anti-rheumatic drugs.

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Part VII
Cancer

Chapter 16
Agonists and Antagonists: Molecular Mechanisms and Therapeutic Applications

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16.1 Cancer Cell Growth Is Driven by Cell Proliferation and Lack of Apoptosis

The growth and expansion of cells is determined on cell ability to go through DNA synthesis and cell division and on the other hand on apoptotic pathways, leading to cell death. Alterations in normal cell function may cause a disruption of normal cell growth and apoptosis, subsequently resulting in carcinogenesis (Jacobson et al. 1997; Danial and Korsmeyer 2004). Understanding of molecular mechanisms underlying the cell proliferation and death machinery is of great importance for improving the efficacy of targeted therapeutics and overcoming resistance to chemotherapeutic agents. Despite the clinical applications of cell cycle inhibitors, mostly chemotherapeutic agents, there is still an urgent need to develop novel drugs that can specifically target multiple sites and pathways of the cell cycle and apoptosis, while avoiding drug induced cytotoxicity.

During the last decade, adenosine was recognized as a cell signaling molecule which binds to specific cell surface receptors and modulate intracellular signaling, resulting in the regulation of physiological processes. Adenosine binds and activates 4 receptor subtypes, A_1, A_{2A}, A_{2B} and the A_3 (Stiles 1990; Linden 1991). Since most body cells express different adenosine receptors and their activation may lead to opposing effects, selective synthetic agonists were developed that induce specific effects on particular cell types.

In this chapter, we will summarize the current knowledge about the A_3AR target, known to be highly expressed in tumor cells. The development of novel A_3AR agonists and antagonists that specifically target the receptor, initiating signal transduction pathways leading to tumor cell cycle arrest and apoptosis will be presented.

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16.2 Over-Expression of A_3AR Is a Characteristic of Tumor Cells

Broad range of tumor cell lines express A_3AR mRNA and protein however, it is quite difficult to compare it to the expression of normal proliferating cells. Nevertheless, a side by side comparison can be made in human samples comparing tumor versus normal tissue. In tissues derived from patients with colon and breast carcinoma, higher A_3AR mRNA and protein expression levels are found in the malignant vs. adjacent non-neoplastic tissue or normal tissue. Additional analysis revealed that the lymph node metastasis expressed even more A_3AR mRNA than the primary tumor tissue, demonstrating that the A_3AR is directly correlated to the degree of malignancy (Madi et al. 2004; Fishman et al. 2007). Gessi et al. also showed that A_3AR expression level was linearly increased in colon tissue samples while progressing from polyp to adenoma, carcinoma and metastatic colon carcinoma (Gessi et al. 2004). Interestingly, the high A_3AR expression level in the tumor tissues was associated with elevated NF-κB levels (Bar-Yehuda et al. 2008). The latter is known to act as one of the A_3AR gene transcription factors and its up-regulation in tumor tissues may account for the high A_3AR expression levels (Madi et al. 2007). High A_3AR mRNA/protein expression levels were also demonstrated in other solid tumor types such as melanoma, pancreatic and small cell lung carcinoma, as well as hepatocellular carcinoma (HCC) (Madi et al. 2004; Fishman et al. 2007). Recent findings show that the A_3AR is over-expressed in the PBMCs of patients with colon cancer and HCC (Gessi et al. 2004; Bar-Yehuda et al. 2008). Therefore, it may be suggested that receptor expression in the PBMCs reflects receptor status in the remote tumor organ.

Bioinformatics analysis revealed a 2.3-fold increase in the expression of A_3AR in human colon adenoma versus normal colon tissue using microarray analysis (Princeton University database). A search in the CGAP (The Cancer Genome Anatomy project; SAGE Genie; Virtual Northern Legend) based on serial analysis of gene expression revealed that A_3AR was abundant in brain, kidney, lung, germ cells, placenta, and retina but brain, lung, and pancreatic tumors expressed more A_3AR in the malignant than the normal relevant tissues. A search in Expression Viewer (HUGO-Gene Nomenclature Committee/CleanEX) based on expressed sequence tags revealed that the relative expression for A_3AR was 1.6-fold higher in all of the cancer tissues compared with normal tissues (Madi et al. 2004).

16.3 Tumors Respond to A_3AR Agonists by Cell Cycle Arrest and Apoptosis

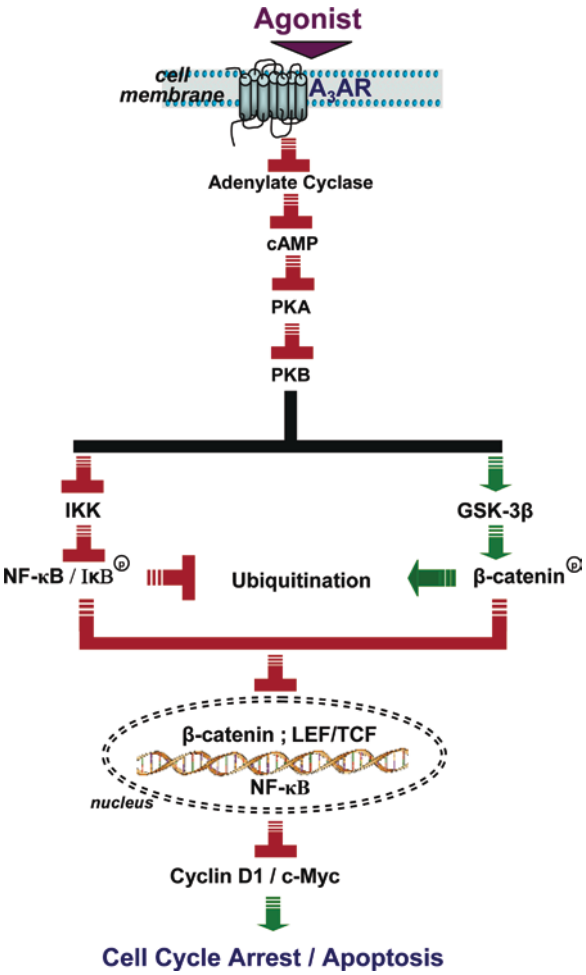
The findings showing up-regulation of the A_3AR in tumor cells prompted studies which led to the utilization of this receptor as a therapeutic target to combat the growth and development of malignant cells. The anti-cancer effect of synthetic A_3AR agonists was explored and the molecular mechanism involved with tumor cell growth inhibition was followed up.

The development and growth of leukemia, lymphoma, melanoma, colon, lung, breast and prostate carcinoma was suppressed *in vitro* upon treatment with IB-MECA, Cl-IB-MECA and thio-Cl-IB-MECA (Bar-Yehuda et al. 2005; Chung et al. 2006; Fishman et al. 2001, 2002a, b, 2003; Fishman and Bar-Yehuda 2003; Kim et al. 2008; Lee et al. 2005; Madi et al. 2003; Merighi et al. 2005a,b; Merimsky et al. 2003; Ohana et al. 2001, 2003; Panjehpour and Karami-Tehrani 2004, 2007). *In vivo* studies in syngeneic murine models showed that IB-MECA suppressed tumor growth inhibition in B16-F10 melanoma, CT-26 colon carcinoma and in an orthotopic model of N1S1 hepatocellular carcinoma (Bar-Yehuda 2008; Fishman et al. 2001, 2002a, 2003; Harish et al. 2003; Madi et al. 2003; Merimsky et al. 2003; Ohana et al. 2001, 2003). Marked tumor growth inhibition was also observed in xenograft mouse models of HCT-116 colon carcinoma and PC-3 prostate carcinoma (Bar-Yehuda et al. 2005; Fishman et al. 2003, 2004; Ohana et al. 2003). The anti-cancer effect was counteracted both *in vitro* and *in vivo* by the selective A_3AR antagonist, MRS1523, demonstrating that tumor growth inhibition is mediated via the A_3AR (Fishman et al. 2003, 2004; Madi et al. 2003; Merighi et al. 2005; Ohana et al. 2003; Panjehpour and Karami-Tehrani 2004, 2007). Interestingly, in a xenograft model and in cell culture studies of breast carcinoma cells, IB-MECA and thio-Cl-IB-MECA suppressed tumor growth, however the effect was not A_3AR mediated due to lack of receptor expression (Chung et al. 2006; Lu et al. 2003).

Mechanistically, the agonists induced down-regulation of cyclin D1 and c-Myc resulting in cell cycle arrest in the G0/G1 phase (Bar-Yehuda et al. 2005; Fishman et al. 2000, 2002a, 2003, 2004; Harish et al. 2003; Kim et al. 2008, Lee et al. 2005; Madi et al. 2003, 2004). Interestingly, two signaling pathways related to Wnt and NF-κB are known to control the expression levels of cyclin D1 and c-Myc (Joyce et al. 2001; Karim et al .2004; Smalley and Dale 1999). Upon treatment with the agonists, cAMP levels are decreased followed by down-regulation of PKA and subsequent decrease in PKB/Akt (known to be phosphorylated by PKA) (Chan et al. 1999; Olah and Stiles 2000; Murga et al. 2000). Modulation of the Wnt and the NF-κB pathways take place down-stream to PKA and PKB/Akt kinases (Hino et al. 2005; Kim and Chung 2002; Li et al. 2006; Vermeulen et al. 2003).

A key protein in the Wnt signaling pathway is glycogen synthase kinase-3β (GSK-3β), is phosphorylated by PKA and PKB/Akt. In quiescent cells, GSK-3β suppresses mammalian cell proliferation and survival by phosphorylating the cytoplasmic protein, β-catenin, which in its phosphorylated form is sorted to ubiquitination. In tumor cells the Wnt signaling pathway is highly activated and GSK-3β fails to phosphorylate β-catenin. The latter then accumulates in the cytoplasm and subsequently translocates to the nucleus where it associates with LEF/TCF to induce transcription of cyclin D1 and c-myc (Ferkey and Kimelman 2000; Novak and Dedhar 1999). PKB/Akt is also known to control the NF-κB signaling pathway by phosphorylating down-stream IKK, which subsequently phosphorylates IκB, thereby releasing NF-κB from its complex. Similar to β-catenin, NF-κB translocates to the nucleus, where among other genes, it induces the transcription of c-Myc and cyclin D1 (Joyce et al. 2001; Madrid et al. 2001; Perkins 2007).

De-regulation of these two pathways was observed in melanoma, colon, prostate, lung, breast and hepatocellular carcinoma *in vitro* and more importantly in tumor lesions derived from agonists' treated animals (Bar-Yehuda et al. 2008; Chung et al. 2006; Fishman et al. 2002a, 2003, 2007; Madi et al. 2004). A point to note is that Cl-IB-MECA induced apoptosis of hepatocellular carcinoma *in vivo*, evidenced by immuno-histochemistry TUNEL staining and by up-regulation of the anti-apoptotic genes caspase-3, Bad and Bax. This tumor type was the most responsive to the treatment of Cl-IB-MECA among all other tumors (Bar-Yehuda et al. 2008) (Scheme 16.1).



Scheme 16.1 Anti-cancer effect of A_3AR agonists – De-regulation of the NF-κB and the Wnt signaling pathways, resulting in cell cycle arrest and apopotosis of cancer cells

An indirect immunomodulatory effect was also observed in the syngeneic CT-26 colon carcinoma and B16-F10 melanoma tumor bearing mice, treated with IB-MECA and Cl-IB-MECA, respectively. The drug agonists induced up-regulation of Interleukin-12 and increased the activity of NK cells (Fishman et al. 2004; Ohana et al. 2003).

A_3AR fate upon treatment with IB-MECA or Cl-IB-MECA was extensively studied. *In vitro* studies revealed that internalization/recycling events that play an important role in turning on/off receptor-mediated signal transduction pathways, take place. In melanoma cells, A_3AR was rapidly internalized to the cytosol and "sorted" to the endosomes for recycling and to the lysosomes for degradation. Receptor distribution in the lysosomes was consistent with the down-regulation of receptor protein expression and was followed by mRNA and protein re-synthesis (Madi et al. 2003). In addition, in melanoma, prostate cancer and HCC tumor lesions derived from IB-MECA or Cl-IB-MECA-treated mice, A_3AR was down-regulated shortly after treatment. In the tumor lesions derived from prostate carcinoma bearing animals, A_3AR was tested also after 18 h and results indicate that receptor was fully expressed (Fishman et al. 2003, 2004; Madi et al. 2003; Ohana et al. 2003). This analysis was carried out after chronic treatment with the agonist, demonstrating that no tachyphylaxis occurred and the target is valid.

It is well established that NF-κB and the upstream kinase PKB/Akt are highly expressed in chemo-resistance tumor cells and play a major role in hampering the apoptotic pathway (Fahy et al. 2004; Wang and Cassidy 2003). Since CF101 has been shown to down-regulate PKB/Akt and NF-κB protein expression levels, it was assumed that its combination with chemotherapy will enhance the anti-tumor effect of the cytotoxic drug. IB-MECA was found to enhance the anti-cancer effect of cyclophosphamide and 5-fluorouracyl (5-FU) in experimental animal models of melanoma and colon carcinoma, respectively (Bar-Yehuda 2005; Fishman et al. 2007; Ohana et al. 2003). Downregulation of PKB/Akt, NF-κB, cyclin D1, and up-regulation of caspase-3 protein expression level were observed in cells and tumor lesions upon treatment with a combination of IB-MECA and 5-FU (Bar-Yehuda 2005). Moreover, in mice treated with the combined therapy, myelotoxicity was prevented as was evidenced by normal white blood cell and neutrophil counts. These results support the notion that IB-MECA potentiates the cytotoxic effect of 5-FU, thus preventing drug resistance (Bar-Yehuda et al. 2002; Fishman et al. 2000, 2002b, 2003; Merimsky et al. 2003). The myeloprotective effect of IB-MECA grants the molecule an added value and suggests its development as a supportive treatment to chemotherapy.

16.4 Hypoxia

The ability to maintain O_2 homeostasis is essential to the survival of all invertebrate and vertebrate species. It is appreciated that all nucleated cells in the human body sense O_2 availability (hypoxia) that is either acute or chronic in duration

(Semenza 2000). As in other physiological systems, adaptive responses to acute changes in O_2 concentration (lasting from seconds or less to minutes) principally occur as a result of alterations (e.g., involving phosphorylation or redox state) of preexisting proteins, whereas chronic changes in O_2 concentration (lasting from minutes to hours or more) principally occur as a result of alterations in gene expression. Not only is O_2 homeostasis essential for survival, but also hypoxia plays an important role in the pathogenesis of major causes of mortality, including cancer, cerebral and myocardial ischemia, and chronic heart and lung diseases (Semenza 1999; Michiels 2004; Maxwell 2002; Hockel and Vaupel 2001).

Most solid tumors develop regions of low oxygen tension because of an imbalance in oxygen supply and consumption. Clinical and experimental evidence suggests that tumor hypoxia is associated with a more aggressive phenotype (Hockel and Vaupel 2001; Vaupel 2008). Hypoxic tumor cells are resistant to conventional chemotherapy and radiotherapy. It is therefore rational to target the hypoxic regions of tumors or disrupt events initiated by hypoxia (Melillo 2004).

Growing evidence from experimental and clinical studies points to the fundamental, pathophysiologic role of hypoxia in solid tumors. In order to survive, humans have to be able to extract oxygen from the atmosphere and make it available to their cells where it is utilised for essential metabolic processes. Hypoxia describes the situation when the body is not receiving quite as much oxygen as it requires. Hypoxia is the result of an imbalance between the supply and consumption of oxygen. It is defined as a partial lack of oxygen. If steps are not taken to increase the supply of oxygen, it switches to anoxia, which is the complete absence of oxygen. Clinical investigations carried out over the last 15 years have clearly shown that the prevalence of hypoxic tissue areas is a characteristic pathophysiological property of solid tumors. As the oxygen concentration decreases with increasing distance from the capillary, both cell proliferation rates and drug concentration decrease. These two factors lead to resistance to anticancer drugs; firstly, because the majority of anticancer drugs are only effective against rapidly proliferating cells; and secondly, because chemotherapy drugs have to reach the tumour cells from the blood vessels (Bertout et al. 2008).

16.5 Adenosine in Hypoxia

One of the major features of solid tumors and even small deposits of tumor tissue is deficiency in the level of oxygen, because of an inadequate vascular supply. The adenosine elevation in response to hypoxia is not exclusive to tumor tissues, but, in this context, the adenosine elevation is localized to the tumor microenvironment, since the surrounding tissue is normally oxygenated. Adenosine is generated mainly by two enzymatic systems: intra- or extracellularly localized 5′-nucleotidases and cytoplasmic S-adenosylhomocysteine hydrolase. The processes of adenosine elimination in the cell involve reactions catalyzed by adenosine deaminase and adenosine kinase (Shryock and Belardinelli 1997) yielding inosine or 5′-AMP,

respectively. The reaction of phosphorylation predominates when adenosine occurs at a low physiological concentration (<1 μM) whereas adenosine deaminase is activated at higher concentrations of the substrate (>10 μM).

The accumulation of adenosine in hypoxia is at least partially explained by hypoxia-mediated regulation of enzymes that are involved in adenosine metabolism: (i) adenosine kinase (Decking et al. 1997) and (ii) 5′-nucleotidase (Headrick and Willis 1989; Kobayashi et al. 2000; Thompson et al. 2004). In particular, adenosine can be generated extracellularly through the hydrolysis of released nucleotides by ecto-5′-nucleotidases (Dunwiddie et al. 1997) or can be produced in the cytosol and transported to the extracellular space (Higgins et al. 1994).

1. Adenosine kinase, which rephosphorylates adenosine to convert it to AMP, was shown to be inhibited by hypoxia (Decking et al. 1997). In addition, extracellular adenosine concentrations may be further potentiated by preventing reutilization through hypoxic inhibition of adenosine deaminase (Sitkovsky et al. 2004; Kobayashi et al. 2000). In particular, first, it has been demonstrated that intracellular adenosine levels due to inhibition of adenosine kinase-dependent metabolism of adenosine to AMP would decrease the transcellular adenosine gradient, thereby decreasing flux through bidirectional equilibrative nucleoside transporters and, thus, elevating extracellular adenosine levels during hypoxia (Decking et al. 1997). Second, transcriptional repression of equilibrative nucleoside transporters decreases overall equilibrative adenosine transport capacities, thereby decreasing intracellularly directed adenosine transport (Eltzschig et al. 2005). It is reasonable that both mechanisms could function during hypoxia and, from this perspective, contribute synergistically to elevate extracellular adenosine during hypoxia and to increase the levels of free adenosine in the tumor extracellular fluid (Blay et al. 1997). In particular, it has been shown, using microdialysis of tumors growing *in vivo*, that adenosine concentrations in the tumor interstitial fluid are 20–30-fold higher than in the adjacent connective tissue. The concentration of adenosine measured in tumor extracellular fluid is of 1–10 μM (Blay et al. 1997).

2. The accumulation of adenosine in hypoxic tissues can also be explained by the hypoxia-mediated upregulation of 5′-nucleotidase activity, an enzyme that converts AMP to adenosine, which results in the accumulation of extracellular adenosine (Sitkovsky et al. 2004).

The levels of extracellular adenosine could increase step-wise up to micromolar levels as the outcome of the transport and/or diffusion of intracellular adenosine, formed from the large pools of intracellular ATP in hypoxic conditions (Sitkovsky et al. 2005, 2008). Hypoxia can upregulate an adenine nucleotide-metabolizing ecto-enzyme cascade comprising ecto-ATP apyrase (CD39) and CD73 (Synnestvedt et al. 2002).

An alternative potential source of extracellular adenosine is the adenosine-3′,5′-cyclic adenosine monophosphate (cAMP), which after being released from cells can be subsequently converted to adenosine (Brundege et al. 1997).

Furthermore, hypoxia increases extracellular adenosine half-life (Eltzschig et al. 2005).

In conclusion, hypoxia appears to induce a program which shifts the cellular phenotype toward an increase in intracellular adenosine.

16.6 Tumor Cells in Hypoxia: Hypoxia-Inducible Factor-1, HIF-1

A major goal in developing new cancer chemotherapeutics is to identify and target biological processes that differ between normal and malignant cells. Ideally, therapeutics should be directed against pathways that have limited redundancy and that are required for development by a broad range of tumors. To survive under hypoxic conditions, tumor cells run numerous adaptive mechanisms, such as glycolysis, glucose uptake, and survival factor up-regulation (Hockel and Vaupel 2001). Hypoxic adaptation involves gene induction via which up-regulates ≈60 genes by binding to 5′-RCGTG-3′ sequences in hypoxia response elements (Semenza 2002). Cancer biologists are therefore becoming increasingly interested in the hypoxia-inducible factor (HIF) transcriptional system. This follows the recognition that HIF is upregulated across a broad range of cancers and is involved in key aspects of tumor biology such as angiogenesis, invasion and altered energy metabolism. Hypoxia-inducible factor (HIF)-1 is a transcription factor that functions as a master regulator of oxygen homeostasis (Semenza 2003, 2008).

HIF-1 is a heterodimer composed of an inducibly-expressed HIF-1α subunit and a constitutively-expressed HIF-1β subunit (Epstein et al. 2001). HIF-1α and HIF-1β mRNAs are constantly expressed under normoxic and hypoxic conditions (Wiener et al. 1996).

The unique feature of HIF-1 is the regulation of HIF-1α expression: it increases as the cellular O_2 concentration is decreased (Minchenko et al. 2002; Semenza 2000). During normoxia, HIF-1α is rapidly degraded by the ubiquitin proteasome system, whereas exposure to hypoxic conditions prevents its degradation (Minchenko et al. 2002). The enzymatic hydroxylation of proline 564 of HIF-1α controls the turnover of the protein by tagging it for interaction with the von Hippel Lindau (VHL) protein (Ivan et al. 2001; Jaakkola et al. 2001; Yu et al. 2001). When cells are hypoxic, the proline residue is not hydroxylated and HIF-1α protein accumulates. The VHL protein forms a multiprotein complex that acts as the ubiquitin ligase that targets HIF-1α for degradation. The effect of hypoxia on Pro-564 hydroxilation can be mimicked by transition metals like cobalt, iron chelators and by inhibitors of the prolyl hydroxylase enzymes (Ivan et al. 2001; Jaakkola et al. 2001).

HIF-1α expression and activity are also regulated by phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signal transduction pathways (Zhong et al. 2000; Semenza 2002).

A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis (Hopfl et al. 2004; Welsh and Powis 2003; Maynard and Ohh 2007). Immunohistochemical analyses have shown that HIF-1α is present in higher levels in human tumors than in normal tissues (Zhong et al. 1999). In particular, the levels of HIF-1 activity in cells are correlated with tumorigenicity and angiogenesis in nude mice (Carmeliet et al. 1998). Tumor cells lacking HIF-1 expression are markedly impaired in their growth and vascularization (Jiang et al. 1997; Maxwell et al. 1997; Ryan et al. 1998; Kung et al. 2000). Therefore, since HIF-1α expression

and activity appear central to tumor growth and progression, HIF-1 inhibition becomes an appropriate anticancer target (Semenza 2003, 2007; Kung et al. 2000; Ratcliffe et al. 2000).

16.7 HIF-1 and the A_3 Receptor

Hypoxia creates conditions that, on one hand, are conducive to the accumulation of extracellular adenosine and, on the other hand, stabilize hypoxia-inducible factors, such as HIF-1α (Linden 2001; Sitkovsky et al. 2004; Fredholm 2003; Hockel and Vaupel 2001; Minchenko et al. 2002; Semenza 2000). HIF-1, the most important factor involved in the cellular response to hypoxia, has been extensively studied this last decade. However, despite the substantial number of investigations into HIF-1, many secrets about its function remain to be revealed.

The actions of adenosine are most prominent in tissues where oxygen demand is high and there is reduction in oxygen tension, that is within solid tumors. In particular, it is recognized that significant levels of adenosine are present in the extracellular fluid of solid tumors (Blay et al. 1997), suggesting a role for this nucleoside in tumor growth. Adenosine mediates its effects through interaction with four adenosine receptor subtypes. Interestingly, it has been demonstrated that the A_3 adenosine receptors are overexpressed in several cancer cell lines as well as in cancer tissues in comparison to normal mucosa (Merighi et al. 2001; Gessi et al. 2001, 2004, 2007). In cancer, the rapid growth of solid tumor frequently results in poor vascularization which creates substantial regions of hypoxia and ischemia that are conducive to adenine nucleotide breakdown, responsible for the adenosine release.

Recent studies support a prominent role for the A_3 receptor as a mechanism to amplify HIF-1 signaling under hypoxic conditions.

It has been specifically focused on responses to chronic hypoxia that involve adenosine-induced changes in the transcription regulator HIF-1 expression. In particular, it has been investigated the correlation between adenosine receptor stimulation and/or blockade and HIF-1α expression modulation in hypoxia. It has been demonstrated that adenosine is able to increase HIF-1α protein expression in response to hypoxia in a dose- and time-dependent manner in human melanoma, glioblastoma and tumor colon cells (Merighi et al. 2005, 2006, 2007). These results indicate that the cell surface A_3 adenosine receptor transduces extracellular hypoxic signals into the cell interior.

In many types of cancers, the HIF-1 pathway is not only activated by hypoxia, it is also induced by a wide range of growth-promoting stimuli and oncogenic pathways. Increased HIF-1α protein synthesis through the activation of Akt or MAPKinase pathways is a common theme accounting for the up-regulation. To evaluate how A_3 receptor accumulates HIF-1α in hypoxia, it has been investigated the signaling pathway generated by A_3 receptor stimulation. It has been found that p44/p42 and p38 MAPKinase activity is required for the HIF-1α expression increase induced by A_3 receptor activation in melanoma and glioblastoma cells and p44/p42, p38 MAPKinase and pAkt activity in colon carcinoma cells (for review see Gessi et al. 2008) (Fig. 16.1).

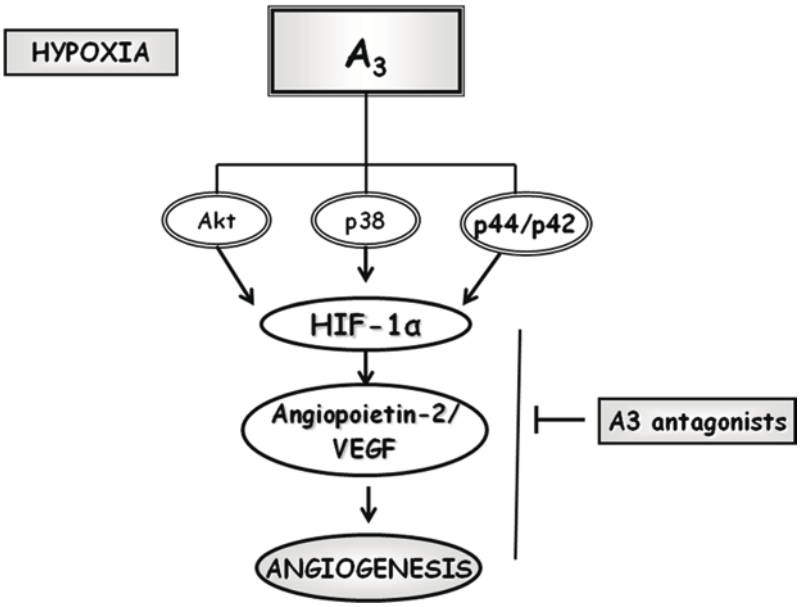


Fig. 16.1 Intracellular mechanism of A_3-mentioned protumoral effect and anticancer effect of A_3 antagonists

16.8 A_3 Receptor and the Angiogenic Response

Angiogenesis is a complex process that involves multiple gene products expressed by different cell types (Conway et al. 2001).

Blockade of angiogenesis is useful in tumor therapy (Ferrara and Kerbel 2005; Menakuru et al. 2008). Over the last decade, the most extensively examined proangiogenic molecule has been VEGF, a secreted protein that, through activation of tyrosine kinase receptors, promotes key events in angiogenesis that include increases in vascular permeability, and stimulation of endothelial cell proliferation and migration (Ferrara 2003).

There is now strong evidence that adenosine, in addition to controlling oxygen delivery acutely by regulating vascular tone, serves a long-term role by enhancing vascular growth in areas with reduced oxygen tension (Adair 2005). Thus, adenosine in physiologically relevant concentrations can stimulate migration and proliferation of endothelial cells. Interestingly, adenosine acting through its receptors also regulates endothelial cell function and promotes angiogenesis. As with VEGF, adenosine-promoted angiogenesis may be considered beneficial in contexts such as wound healing and myocardial ischemia, or detrimental in disease states such as cancer or in

retinopathy of prematurity (Adair 2005). Stimulation of angiogenesis may be achieved by stimulating A_{2A} and/or A_{2B} receptors and recently also A_1 in a variety of cells including endothelial, smooth muscle cells, bronchial epithelial cells, macrophages, monocytes through both HIF-independent and HIF-dependent pathways (Feoktistov et al. 2002, 2004; Montesinos et al. 2004; Allen-Gipson et al. 2006; Clark et al. 2007; for review see Auchampach 2007). However there is evidence for a role of the A_3 receptor and HIF-1 in angiogenesis when cancer cells are studied (Merighi et al. 2005, 2006, 2007). In particular, adenosine, released during tissue injury, ischemia and tumor growth, is able to promote angiogenesis by stimulation of angiopoietin-2 secretion via A_3 receptors (Feoktistov et al. 2003; Merighi et al. 2005). It has been proposed that the effect of VEGF on new capillary formations is facilitated by the concomitant stimulation of A_{2B} and A_3 receptors that induce the expression of angiopoietin-2 (Feoktistov et al. 2003). Furthermore, activation of the A_3 adenosine receptor subtype in glioblastoma and colon cancer cells stimulates VEGF expression (Merighi et al. 2006, 2007), whereas this receptor subtype promotes VEGF down-regulation in PC12 pheochromocytoma cells (Olah and Roudabush 2000). Furthermore, the activation of A_3 receptors results in increased expression of angiopoietin-2 in mast and melanoma cells (Feoktistov et al. 2003; Merighi et al. 2005).

Although adenosine may contribute rather little to the increase in VEGF induced by hypoxia, it may contribute as much as 50% to angiogenesis (Adair 2005; Auchampach 2007). This could mean that adenosine acts also independently of VEGF, something that is not unlikely given the involvement of multiple cell types and multiple angiogenetic factors.

Recent studies indicate that pharmacologic inhibition of HIF-1α and particularly of HIF-regulated genes, that are important for cancer cell survival, may be useful to improve cancer treatment outcomes. However, the use of HIF-1 inhibitors as anticancer agents must occur within the conceptual framework of combination therapy, as administration of multiple agents simultaneously is essential for the successful treatment of human cancer (Semenza 2007). In this regard, it should be underlined that the A_3 adenosine receptor antagonists are able to block HIF-1α, Angiopoietin-2 and VEGF protein expression accumulation in hypoxia, indicating a new approach for the treatment of cancer, based on the cooperation between hypoxic and adenosine signals.

16.9 A_3 Receptor and the Immunosuppression

The resistance of many human cancers to immunotherapies has been attributed to the presence of immunosuppressive molecules located in tumor areas. Adenosine is present at elevated levels in hypoxic tissues due to an increased intracellular production and extracellular accumulation, as described above. This nucleoside activates cell surface receptors on T and NK cells that mediate cellular immune responses to tumor cells. It is well established that T cells recognize and destroy

cancer cells *in vitro* whilst fails to do so *in vivo*. This mechanism of tumor protection has been attributed to the immunosuppressive role played by adenosine that inhibits T lymphocytes activation, including adhesion to tumor cells and cytotoxic activity. Blay and coworkers have been the first to hypothesized that elevated levels of adenosine in solid tumors might be responsible for impaired destruction of tumor cells by immune effector cells. They identify the A_3 receptor subtype as the one responsible for these effects suggesting a role for A_3 antagonists in the immunotherapies of tumors (for review see Hoskin et al. 2008). Conversely, recently it has been reported that genetic deletion of immunosuppressive A_{2A} and A_{2B} receptors can prevent the inhibition of anti-tumor T cells thus suggesting a role for A_2 antagonists to improve full tumor rejection (Sitkovsky and Ohta 2005, Ohta et al. 2006). As described in detail in the chapter on immune cells (lymphocytes paragraph) of the present book the identification of adenosine receptor subtypes through which adenosine exerts its inhibitory effects on cell-mediated anti-tumor immune responses need further investigations at least for what concerns A_3 receptors and will allow us the development of specific pharmacologic approach to improve tumor rejection by antitumor cells.

16.10 Conclusions

To conclude, the efficacy of Cl-IB-MECA in several tumor animal models, especially HCC, prompted the introduction of this molecule into a program of pre-clinical and clinical studies. The excellent safety profile in pre-clinical animal studies and human Phase I in healthy subjects led to the initiation of Phase I/II studies in patients with HCC, currently ongoing.

On the other hand, from the results summarized in this chapter, it appears evident that also the future for generation of A_3 receptor antagonists in the treatment of human cancer can be considered promising, even though the clinical efficacy still remains to be demonstrated.

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