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Adenosine Receptors: New Opportunities for Future Drugs

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Abstract—This review summarises current knowledge on adenosine receptors, an important G protein-coupled receptor. The four known adenosine receptor subtypes A_1, A_{2A}, A_{2B}, and A_3 are discussed with special reference to the opportunities for drug development.

Introduction

The potential of adenosine receptors as drug targets was first reviewed in 1982.[1] There has been a major review in 1992 of progress over the subsequent ten year period.[2] Significant progress has occurred over the last few years. The regulatory role for adenosine (**1**) (Figure 1) implicates adenosine based drugs as therapeutic targets. An improved understanding of the physiology, pharmacology and molecular biology of adenosine and adenosine receptors has taken place in recent years. This understanding is fundamental to the realisation of the therapeutic potential for this nucleoside and provides a solid foundation for the continuation of active research in the adenosine field. The endogenous nucleoside, adenosine, is ubiquitous in mammalian cell types. Adenosine is related both structurally and metabolically to the bioactive nucleotides adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and cyclic adenosine monophosphate (cAMP); to the biochemical methylating agent *S*-adenosyl-L-methione (SAM); and structurally to the coenzymes NAD, FAD and coenzyme A; and to RNA. Together adenosine and these related compounds are important in the regulation of many aspects of cellular metabolism.

Key words: Adenosine; receptors; structure–activity; review; molecular modelling.
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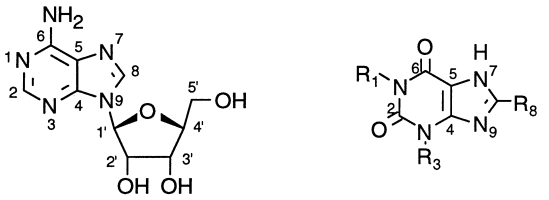
Adenosine mediates many of its physiological effects via cell surface receptors. The receptors for adenosine and adenine nucleotides were first proposed to be classified as the P_1 and P_2 purinergic receptors.[3] That classification was based on (i) the relative potencies of adenosine and adenine nucleotides; (ii) the sensitivity to antagonism by methylxanthines; and (iii) modulation of activity of adenylate cyclase. The P_2 receptor classification was characterised by (i) the rank order of potency: adenine nucleotides > adenosine; (ii) insensitivity to antagonism by methylxanthines; and (iii) induction of prostaglandin synthesis.

The existence of two subtypes of P_1 receptors was independently proposed.[4,5] Evidence for one proposal was based on the observation of either increased stimulation or increased inhibition of cAMP formation by adenosine analogues compared to adenosine.[4] The receptor subtype which mediated inhibition of formation of cAMP was termed the A_1 subtype and the receptor subtype which mediated stimulation of formation of cAMP was termed the A_2 subtype. Evidence for the other proposal was based on the observation of two profiles for the relative effects of adenosine, N^6-(phenylisopropyl)adenosine and 5'-*N*-ethylcarboxamidoadenosine on adenylate cyclase activity.[5] These receptor subtypes were termed R_a (activation of adenylate cyclase activity) and R_i (inhibition of adenylate cyclase activity), respectively. It is accepted that A_1 and R_i correspond to the same receptor subtype, as do A_2 and R_a.

There is an obvious need for consistency in nomenclature and classification of receptors in order to minimise



1 **2**

Figure 1. The structures of adenosine (**1**) and xanthine (**2**).

confusion in the literature. This need was recognised by the International Union of Pharmacology (IUPHAR) Committee on Receptor Nomenclature and Drug Classification, which has suggested a set of rules for nomenclature of receptors and receptor subtypes.[6] They state that a 'completely' defined receptor would include both molecular and pharmacological information about that receptor. This definition includes a known endogenous ligand, a unique pharmacological profile (based on agonist and antagonist data), a distinct amino acid sequence, structural type (e.g., G protein-coupled, gated ion channel, etc.) and effector system. Molecular cloning is expected to become the primary means of discovery of new receptors and receptor subtypes, as opposed to the classical manner of pharmacological discovery, and hence such a 'completely' defined receptor is a realistic concept. Based on these nomenclature and classification rules P_1 receptors are also named adenosine receptors (after the endogenous ligand), while the subtypes are named the A_1, A_{2A}, A_{2B} and A_3 subtypes, the subscripts (1, 2A, 2B and 3) representing classification neutral labels.[7,8] Each of the subtypes has been characterised by molecular cloning, agonist activity profile, antagonist activity profile, G protein-coupling and effector systems. A putative new adenosine receptor subtype, named A_4, has been proposed.[9] The basis for this proposal was pharmacological evidence of agonist binding profiles. This characterisation alone is insufficient for definition of this receptor as a new adenosine receptor subtype as the requirement for structural evidence from molecular cloning is lacking.[7] Other evidence disputes the classification of the proposed A_4 subtype as a distinct adenosine receptor subtype.[10] The existence of further adenosine receptor subtypes, as yet undiscovered, is however plausible. If discovered they will easily be accommodated by this nomenclature and classification system.

Characterisation of adenosine receptor subtypes

Four adenosine receptor subtypes have now been characterised pharmacologically, structurally and functionally, Table 1. The A_1, A_{2A} and A_{2B} subtypes were initially discovered and classified in the classical manner (i.e., by a study of agonist pharmacology). Evidence from recent cloning, sequencing and expression of each of these subtypes has provided structural and functional confirmation of their original classification as distinct adenosine receptor subtypes. In contrast, the A_3 receptor subtype was discovered by molecular biology studies, this was then followed by classical pharmacological studies. All four adenosine receptor subtypes have recently been cloned from a variety of mammals, including humans (Table 1).

Molecular biology has made significant contributions to adenosine receptor characterisation[11] allowing determination of actual receptor protein primary sequences, which is fundamental to studying receptor structure, function and regulation at the molecular level.[11] Subtype specific probes for mRNA allow characterisation of the receptor subtype makeup of a particular cell or tissue, this method represents a vast improvement over classical pharmacological techniques. Preparation of pure receptor subtype populations, both wild type and mutant (using site-directed mutagenesis), in identical cellular environments is also possible with cloning techniques. This enables the study of, for example, G protein-coupling and regulation, effector systems, molecular interactions involved in ligand binding and pharmacological profiles of ligands, without the complications caused by the presence or interference of other receptor subtypes.

The variability of specific subtype pharmacology in multiple species means that the pharmacology of other species may not always be relevant to human pharmacology. With molecular clones available from multiple species, an appropriate animal model may be rationally selected for the evaluation of potential therapeutics for later human use.[11]

Analysis of the amino acid sequences of the cloned receptors demonstrates that they all fit the seven transmembrane spanning domain structural motif, which is the model for all G protein-coupled receptors.[2,12,13] This structural motif encompasses seven domains, each ~22–26 hydrophobic amino acids, which traverse the cell membrane. These domains possess alpha helical secondary structure. The extracellular domains of the receptor protein comprise the N-terminus and three extracellular loops connecting the transmembrane domains (EI–III), while the cytoplasmic or intracellular domains of the receptor protein comprise the C-terminus and similarly three intracellular loops (CI–III), Figure 2. Generally the amino acid sequences for G protein-coupled receptors demonstrate high overall homology for the same receptor subtype in different species, typically 85–95%.[7] In the transmembrane spanning regions homology is highest, while less sequence conservation is observed for other regions. The overall sequence homology for adenosine receptor subtypes,

Table 1. Characterisation of adenosine receptor subtypes[7,11–13,16,126–132]

	Adenosine A_1 receptor	Adenosine A_{2A} receptor	Adenosine A_{2B} receptor	Adenosine A_3 receptor
Tissue distribution	Brain (cortex, hippocampus, cerebellum, thalamus), spinal cord, testis, white adipose tissue, heart, kidney	Brain (striatum, nucleus accumbens, tuberculum olfactorium), heart, lungs, thymus, spleen, white adipose tissue	Pars tuberalis, large intestine, bladder	Species dependent; Rat: testis ≫ lung, kidney, heart > brain Human: lung, liver, placenta ≫ brain, aorta, kidney > testis > heart Sheep: lung, spleen > brain, testis
Cloning	Canine, rat, human, bovine, rabbit, mouse; 326–328 amino acids[39,133–142]	Canine, rat, human, guinea pig, mouse; 409–412 amino acids[39,133,143–147]	Rat, human, mouse; 332 amino acids[39,148–150]	Rat, human, sheep; 317–320 amino acids[89,90,98,151]
Agonist Profile	High (0.3–3 nM) CPA, CHA, R-PIA, ADAC Intermediate (3–30 nM) NECA, 2-CADO, ADO Low (30–350 nM) S-PIA, DPMA Very low (> 350 nM) CV1808, CGS21680, APEC	High (1–20 nM) NECA, CGS21680, APEC, ADO Intermediate (20–200 nM) CADO, CV1808, R-PIA, ADAC Low (200–500 nM) CPA, CHA, S-PIA	High (0.5–5 μM) NECA Intermediate (5–20 μM) 2-CADO, ADO, R-PIA Low (20–100 μM) S-PIA Very low (> 100 μM) CGS21680, CV1808	High (< 10 nM) APNEA, N^6-benzyl NECA Intermediate (10–30 nM) NECA, R-PIA, I-ABA Low (100–1000 nM) CGS21680 Very low (> 1 μM) ADO
Antagonist Profile	High (0.5–2 nM) DPCPX, XAC Intermediate (2–200 nM) CPT, 8-PT, CGS15943 Low (1–20 μM) Theophylline, 8-*p*SPT, IBMX, KF17387 Very low (> 20 mM) Caffeine, DMPX, CSC	High (20–100 nM) XAC, CSC, KF17837, CGS15943, SCH58261, ZM241385 Intermediate (0.2–2 μM) CPT, DPCPX, 8-PT Low (2–20 μM) DMPX, 8-*p*SPT, IBMX, Theophylline Very low (> 30 μM) Caffeine	High (20–100 nM) XAC, DPCPX, 8-PT, CGS15943 Intermediate (0.5–10 μM) 8-*p*-SPT Low (10–20 μM) Theophylline, DMPX, IBMX Very low (> 30 μM) Caffeine, KF17837	High (1–20 nM) BWA522 Very low (> 100 μM) 8-PT, XAC, IBMX, DPCPX, theophylline
Structural type Effector system	G Protein-coupled: $G_{i(1-3)}$, G_o, $G_{q/11}$ Adenylate cyclase: ↓ cAMP Phospholipase C: ↑↓ IP_3, ↑ Ca^{2+} Ion channels: ↑ K^+, ↓ Ca^{2+}	G protein-coupled: G_s Adenylate cyclase: ↑ cAMP	G protein-coupled: G_s Adenylate cyclase: ↑ cAMP	G protein-coupled: $G_{i(2,3)}$,$G_{q/11}$ Adenylate cyclase: ↓ cAMP Phospholipase C: ↑ IP_3, ↑ Ca^{2+}

Abbreviations: CPA, N^6-(cyclopentyl)adenosine; CHA, N^6-(cyclohexyl)adenosine; R-PIA, N^6-(*R*-phenylisopropyl)adenosine; S-PIA, N^6-(*S*-phenylisopropyl)adenosine; ADAC, adenosine amine congener; NECA, 5′-*N*-ethylcarboxamidoadenosine; 2-CADO, 2-chloroadenosine; ADO, adenosine; CV1808, 2-phenylaminoadenosine; CGS21680, 2-[[*p*-(2-carboxyethyl)phenethyl]amino]-5′-*N*-ethylcarboxamidoadenosine; APEC, 2-[(2-aminoethylamino)carbonylethylphenethylamino]-5′-*N*-ethylcarboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; XAC, xanthine amine congener; CPT, 8-cyclopentyltheophylline; 8-PT, 8-phenyltheophylline; CGS15943, 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethanesulfonate; 8-*p*SPT, 8-*p*-sulfophenyltheophylline; IBMX, 3-isobutyl-1-methylxanthine; KF17387, 1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine; DMPX, 1,3-dimethyl-7-propylxanthine; CSC, 8-(3-chlorostyryl)caffeine:APNEA, N^6-(2-(4-aminophenyl)ethyl)adenosine; I-ABA, N^6α-(3-iodo-4-aminobenzyl)adenosine; BWA522, 3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine; IP_3, inositol triphosphate; SCH58261, 5-amino-7-(2-phenylethyl)-2-(d-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-c]pyrimidine; ZM241385, (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol).

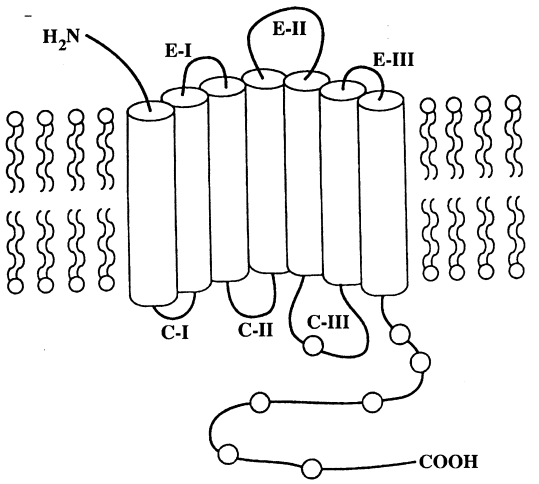


Figure 2. Seven transmembrane spanning domain structural motif of adenosine receptors. The extracellular domains of the receptor protein comprise the N-terminus and three extracellular loops (EI-III), while the intracellular domains of the receptor protein comprise the C-terminus and three intracellular loops (CI-III). (Figure adapted from Jacobson et al.[2])

between species, is presented in Table 2. The homology data is consistent with that expected for G protein-coupled receptors, with the exception of the A_3 subtype, with only 74% primary sequence homology between rat and human or sheep.[14]

The role of highly conserved sequences is structural and/or functional, and most likely such sequences are involved either directly or indirectly in G protein-coupling, to enable signal transduction and/or ligand binding. The lesser homology exhibited for the A_3 subtype is reflected by the significant differences in pharmacology between species, including agonist and antagonist binding, tissue distribution, and diversity of structure with respect to G protein-coupling and effector systems.[7,13,15] The biological response mediated by a particular receptor is additionally influenced by external factors such as receptor density, efficacy and the presence of spare receptors in the locality of the particular receptor.

Table 2. Overall sequence homology for adenosine receptor subtypes, between species[13–15]

Subtype	Overall % sequence homology	
A_1	87	(canine–rat–human–bovine–rabbit)
A_{2A}	82	(rat–canine)
	93	(human–canine)
A_{2B}	86	(rat–human)
A_3	74	(rat–sheep) (81 transmembrane)
	74	(rat–human) (82 transmembrane)
	85	(sheep–human) (92 transmembrane)

In human, the homology among the four subtypes is 30% overall, and 45% in the transmembrane domains.[13] A further breakdown of this data, comparing only two given subtypes at a time, is given in Table 3.[13,15] The data shows a degree of overall homology ranging from 40 to 61% between different adenosine receptor subtypes in a single species, human. A consequence of this high inter-subtype homology, which would be greater for the transmembrane region alone, is demonstrated by the difficulty of designing or developing subtype selective ligands.

Activation of adenosine receptors by adenosine initiates signal transduction mechanisms. These mechanisms are dependent on the receptor associated G protein. Each of the adenosine receptor subtypes has been classically characterised by the adenylate cyclase effector system, which utilises cAMP as a second messenger. The A_1 and A_3 receptors, coupled with G_i proteins inhibit adenylate cyclase, leading to a decrease in cellular cAMP levels, while the A_{2A} and A_{2B} receptors couple to G_s proteins and activate adenylate cyclase, leading to an increase in cellular cAMP levels.

The specificity of subtypes with respect to G protein and associated effector system remains to be determined. The A_1 and A_3 subtypes are associated with effector systems other than adenylate cyclase (Table 1). Most detail is known for the A_1 receptor effector systems, which include activation of phospholipase C and modulation of both potassium and calcium ion channels.[13] The A_3 subtype, in addition to its association with adenylate cyclase, also stimulates phospholipase C and so activates calcium ion channels.[12,16]

Physiological significance of endogenous adenosine

The initial realisation of the physiological significance of adenosine was in 1929.[17] Pronounced physiological effects were observed on both cardiovascular and renal function following administration of adenosine to mammals. The clinical evaluation of adenosine in man proved disappointing due to the short half-life of adenosine,[18] and interest waned in the potential therapeutic applications.

Table 3. Overall sequence homology of human adenosine receptor subtypes[13,15]

Human subtypes	Overall % homology
A_1:A_{2A}	51
A_1:A_{2B}	46
A_1:A_3	50
A_{2A}:A_{2B}	61 (transmembrane 73)
A_{2A}:A_3	43
A_{2B}:A_3	40

The ability to study further the physiological role of endogenous adenosine in a variety of mammalian tissues followed the discovery that the xanthines, caffeine and theophylline, were adenosine antagonists.[19] A cascade of publications on adenosine and adenosine receptors soon followed. Adenosine is now known to regulate a diverse range of physiological functions, to the extent that almost all mammalian organ systems are affected by adenosine.[20] Adenosine is proposed to function as a paracrine homeostatic modulator with a global rather than specific role.[21] The physiological responses to adenosine are complex and depend on the receptor subtype activated, the mammalian species, and the type and metabolic state of the tissue.[21]

The A_1 and A_{2A} receptor subtypes are proposed to play complementary roles in adenosine's regulation of the energy supply:demand balance of cells.[22] A schematic representation of this homeostasis is presented in Figure 3. An increase in the oxygen demand of tissues (exercise) or a decreased oxygen supply (ischaemia, hypoxia) results in an imbalance of the energy supply:demand of tissue. An acute increase in the adenosine levels, due to the metabolism of ATP, follows. Adenosine diffuses from the cell where it acts locally to activate adenosine receptors to decrease the oxygen demand (A_1) or increase the oxygen supply (A_{2A}) and so reinstate the balance of energy supply:demand within the tissue. The actions of both subtypes is to increase the amount of available oxygen to tissues and so protect cells against damage caused by a short term imbalance of oxygen.

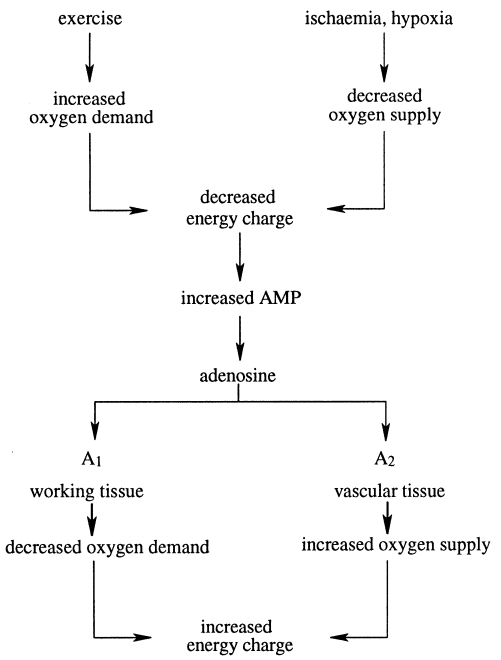


Figure 3. Role of adenosine, adenosine A_1 and A_{2A} receptors in energy supply:demand balance.[22]

The mechanism for tissue specific expression of the A_1 and A_{2A} subtypes is not known, however the receptors are present in nearly all mammalian tissue types and may be coexpressed in the same cell type. A consequence of the global homeostatic function of endogenous adenosine is the multitude of physiological responses mediated by adenosine, presented in Table 4. These responses generally lead to a decrease in the oxygen demand and/or increase in the oxygen supply, reinstating the energy supply:demand balance within the tissue. One of the most important functions of endogenous adenosine is as a cytoprotective agent, preventing tissue damage during traumas such as hypoxia, ischaemia, hypotension and seizure activity.[23–27]

The A_{2B} receptor subtype is a low affinity receptor, adenosine exhibiting activity at this subtype at concentrations greater than 10 μM.[28,29] There is little information on the physiological significance of the A_{2B} subtype, a consequence of the lack of suitably potent and selective ligands for detailed study.[13] This deficiency is also reflected by the means in which A_{2B} receptor clones are pharmacologically identified: not by specific binding of particular ligands, as for the other receptor subtypes, but rather by the lack of specific binding of ligands. However, the expression of A_{2B} subtype clones to give pure populations of this receptor may facilitate future pharmacological characterisation.[11] A speculative role for the A_{2B} receptor has been proposed.[22] It was suggested that the A_{2B} receptor functions during life threatening systems failure, reactivating the heart and brain, in order to resuscitate. This action would override the protective functions afforded by the A_1 and A_{2A} receptors. More recent studies have demonstrated that activation of A_{2B} receptors leads to increases in intracellular calcium concentrations in cultured cells and chloride ion secretion via cAMP in intestinal epithelial cells.[30,31] The latter has implications for the treatment of secretory diarrhea associated with inflammation.[31]

The physiological role for the A_3 receptor is not adequately understood, a consequence of its relatively recent characterisation and a lack of truly selective ligands for in vivo studies. Suitably selective and potent ligands (both agonists and antagonists) are being developed predominantly by radioligand binding studies, however the relationship between the radioligand binding data and selectivity in vivo is not yet established.[32–36] As well, ligands which are already well characterised pharmacologically at the A_1 and A_{2A} receptors are being characterised at the A_3 receptor.[37,38] The availability of cell lines expressing the recombinant A_3 receptor from different species is facilitating

Table 4. The physiological effects mediated by endogenous adenosine in various mammalian tissues, attributed to activation of A_1 and A_{2A} receptor subtypes

System	Physiological effect of endogenous adenosine
CNS	Neuromodulator mediating central inhibitory effects, inhibits release of neurotransmitters,[152] inhibits neuronal firing,[153] CNS depressant: decreases locomotor activity, anticonvulsant, sedation, hypnotic,[57,154–159] antinociceptive,[160] ataxic,[157] mediates respiration[52,161]
CV	Vasodilation, hypotensive,[52,66] antiarrhythmic,[52,67,162,163] negative chronotropic, dromotropic and inotropic effects,[66,67,164,165] inhibits platelet aggregation[166–170] decreases neutrophil function,[171,172] stimulates nitric oxide production by vascular endothelial cells[173]
Kidney	Biphasic modulation of renin release,[68] decreased glomerular filtration rate by vasoconstriction[68]
Adipocytes	Inhibits lipolysis (maintenance of body weight)[174,175]
Immune	Immunosuppressant,[171,176] inhibits lymphocyte proliferation, antiinflammatory: modulating neutrophil function
Liver	Regulates hepatic blood flow,[177] stimulates gluconeogenesis,[178,179]
Stomach	Inhibits gastric secretion[180,181]
Striated/smooth muscle	Relaxation[182,183]

advancements in the study of its physiological role. So far this subtype is implicated in mediating a number of physiological responses.

Binding of the adenosine agonist NECA to mast cells expressing the rat A_3 receptor resulted in increased inositol triphosphate and intracellular calcium concentrations, which potentiated antigen induced secretion of inflammatory mediators (histamine, leukotrienes, cytokines, thromboxanes and proteases).[16] The conclusion from these studies was that the A_3 receptor may play a role in mediating asthmatic attacks and other allergic responses, however other adenosine receptor subtypes have not been ruled out in contributing to the observed responses.[12,39] Using the A_3 selective, high affinity agonist N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine (3-IB-MECA), in vivo studies in mice demonstrated the A_3 receptor mediates a locomotor behavioural depressant effect, possibly centrally mediated.[40] A cardioprotective role for A_3 receptors, activating ischaemic preconditioning, has been proposed based on studies in isolated rabbit heart.[41] N^6-2-(4-aminophenyl)ethyladenosine (APNEA) produced a hypotensive response in the pithed rat, which was attributed to activation of A_3 receptors.[42] It was subsequently demonstrated that this A_3 receptor activation resulted in mast cell degranulation and histamine release, implicating the mast cell with a key role in A_3 receptor mediated hypotension in the rat.[43,44]

Adenosine metabolism and transport

Adenosine is produced in many cell types with a basal concentration in the μM range. Studies in guinea pig and dog heart estimate the concentration of endogenous adenosine in interstitial fluid as between 0.1 and 0.3 μM.[45,46] Figure 4 shows the major pathways responsible for the metabolism (biosynthesis and degradation) and transport of adenosine.[47,48] The biosynthesis of adenosine is primarily by two pathways. The first is a cascading hydrolysis pathway, from 5′-ATP to 5′-ADP to 5′-AMP to adenosine, this can occur both intracellularly and extracellularly. The second is the intracellular enzymatic conversion of *S*-adenosylhomocysteine to adenosine.

Transport of intracellularly produced adenosine out of the cell, where the extracellular cell surface adenosine receptors are located, is primarily by facilitated diffusion through a specific nucleoside transporter protein. The lifetime of adenosine in circulation is in the order of several seconds.[49] This rapid degradation means that adenosine acts locally, close to the site where it first enters circulation.

The elimination of extracellular adenosine is most commonly by: (i) diffusion back into the cell; either facilitated (by a specific nucleoside transporter protein) or active (by an energy requiring nucleoside transporter protein capable of concentrative transport); or (ii)

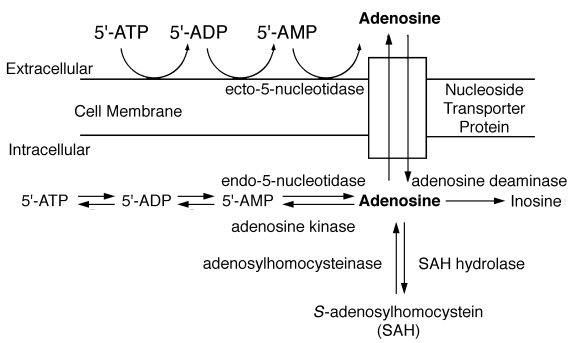


Figure 4. Major pathways of adenosine metabolism and transport.

enzymatic: deaminated by adenosine deaminase to inosine. Intracellular adenosine is either metabolised enzymatically by adenosine deaminase to inosine; or by adenosine kinase, forming 5′-AMP.

Therapeutic potential for drugs acting at adenosine receptors

The diverse physiological functions of adenosine, outlined earlier, highlight the significant benefits of developing therapeutics for the regulation of adenosine receptors. The vast amount of effort invested in adenosine research is driven by the therapeutic potential for drugs which elicit their actions at adenosine receptors. However, the ubiquitous distribution of adenosine receptors in mammalian cell types, the existence of at least four distinct subtypes together with the variability of physiological responses means that exploiting this potential requires agonists and antagonists that are highly selective in their action (with respect to receptor subtype and tissue type) to be of value as therapeutics.

Successful development of therapeutics is far from trivial. Many promising ligands have been identified in the pursuit of therapeutic agents, however side effects due to low selectivity have precluded clinical development. The recent cloning of each of the adenosine receptor subtypes has allowed, and will continue to allow, the study of receptor subtype structure at the molecular level. These studies may identify the discrete differences between subtypes in terms of ligand binding requirements, and may prove fundamental to the successful design of not only potent ligands but also subtype selective ligands.

Adenosine itself can be a useful therapeutic agent when a short acting response is sufficient to achieve the desired tissue state. Selective action at the site of administration can be achieved as a result of the rapid metabolism of adenosine (half-life $\sim$10 s).[49] Adenosine as Adenocard™ is used clinically in the treatment of supraventricular tachycardia.[50,51] The coronary vasodilation produced by adenosine has lead to its use in both diagnosis of coronary stenosis and also as a hypotensive agent during aneuryism surgery.[52,53]

The physiological function of adenosine in the central nervous system (CNS) has been extensively researched during the last two decades. Adenosine has been described as a neuromodulator, possessing global importance in the modulation of the molecular mechanisms underlying many aspects of brain function by mediating central inhibitory effects.[21,54] An increase in neurotransmitter release follows traumas such as hypoxia, ischaemia and seizure activity. These neurotransmitters are ultimately responsible for neural degeneration and neural death, which causes brain damage or death of the individual. The development of adenosine A_1 agonists which mimic the central inhibitory effects of adenosine (and so inhibit neurotransmitter release) may therefore be clinically useful as neuroprotective agents.[55,56] Adenosine has been proposed to be an endogenous anticonvulsant agent, inhibiting glutamate release from excitory neurons and inhibiting neuronal firing.[57] Adenosine agonists therefore have potential clinical applications as antiepileptic agents. Alkylxanthines, which are adenosine antagonists, stimulate the activity of the CNS and have proven to be effective as cognition enhancers.[58,59] This is the joint action of antagonism of the sedative effects caused by adenosine and of increasing cerebral blood flow, thus increasing glucose and oxygen availability to the brain.[21] Selective antagonists may have therapeutic potential in the treatment of various forms of dementia, for example in Alzheimer's disease. The pathological hallmark of Parkinson's disease is the depletion of dopamine in the striatum, as a consequence of the degeneration of the substantia nigra. With the knowledge that adenosine inhibits the release of dopamine from central synaptic terminals and that A_2 agonists reduce locomotor activity it was hypothesised that A_2 antagonists might increase the release of dopamine and consequently improve Parkinsonian symptoms. Theophylline, in low doses, produced significant improvements in symptoms of patients with Parkinson's disease, and was proposed as a safe adjunct in the therapy of Parkinson's disease.[60] The central activities of adenosine are also implicated in the molecular mechanisms underlying sedation, hypnosis, schizophrenia, anxiety, pain, respiration, depression and substance abuse. Drugs acting at adenosine receptors therefore have therapeutic potential as sedatives, muscle relaxants, antipsychotics, anxiolytics, analgesics, respiratory stimulants and antidepressants.[61] An important role for adenosine in the cardiovascular system is as a cardioprotective agent. Levels of endogenous adenosine increase in response to ischaemia and hypoxia, and protect cardiac tissue during and after trauma, a process called ischaemic preconditioning.[62,63] Adenosine agonists thus have potential as cardioprotective agents.[64,65] Adenosine is an antiarrythmic agent, eliciting negative chronotropic and dromotropic effects.[66,67] By either slowing or terminating abnormal cardiac rhythms, it is used to treat supraventricular tachycardias. The short half-life of adenosine specifies a transient response, however this is sufficient to interrupt the electrical impulses through the atrioventricular node and produce the desired response. Development of adenosine agonists may be useful in the management of cardiac arrhythmias. In contrast adenosine antagonists are cardiotonic in ischemic hearts that are producing increased amounts of adenosine. They antagonise the negative chronotropic and dromotropic actions of

adenosine and so increase cardiac output.[61] Adenosine agonists act directly as hypotensive agents through two mechanisms: vasodilation and reduction of cardiac output.[21] Many agonists have been evaluated as hypotensive agents, however side effects, toxicity and/or the existence of already effective antihypertensive agents has precluded clinical development.[49]

Adenosine modulates many aspects of renal function, including renin release, glomerular filtration rate and renal blood flow. It plays a role in mediating the haemodynamic changes associated with acute renal failure.[68] Xanthines, which antagonise the renal affects of adenosine, have potential as renal protective agents. Theophylline has been shown to improve renal function in humans, preventing acute renal failure caused by iodine-containing radiographic contrast media used in X-ray examination. It has also been shown to improve renal function after kidney transplantation.[69] The xanthine antagonist 1,3-dipropyl-8-(3-noradamantyl) xanthine (KW-3902), is currently undergoing clinical trials as a renal protective agent.[70]

Inhaled adenosine induces bronchoconstriction in asthmatics.[71] The mechanism for this modulation is not completely characterised. Binding of the adenosine agonist NECA to mast cells expressing the rat A_3 receptor resulted in increased inositol triphosphate and intracellular calcium concentrations, which potentiated antigen induced secretion of inflammatory mediators (for example histamine, leukotrienes, cytokines, thromboxanes and proteases).[16] Another study in a human mast cell line has demonstrated that agonists acting at the A_{2B} receptor stimulate the release of inflammatory mediators.[72] A role is proposed for adenosine in the aetiology of asthma, suggesting that antagonists (A_3 and/or A_{2B}) may be useful in the treatment of asthma and other allergic responses.[15,72]

Structure–activity relationships for ligands at adenosine receptors

The primary mechanism for studying adenosine receptors, prior to the recent use of molecular biology techniques, has been by pharmacological means. A large number of ligands have been synthesised and evaluated for adenosine receptor binding affinity. Due to the large number of compounds, particularly for the A_1 and A_{2A} subtypes, a quite detailed compilation of structure–activity relationships has been acquired. These extensive structure–activity relationships have enhanced the understanding of the binding domains of adenosine receptors, highlighting the key structural features required for receptor affinity and subtype selectivity. Also, these structure–activity relationships, together with molecular modelling techniques, have been used in the development of a pharmacophore of the ligand binding characteristics.

With the discovery of the A_3 receptor, structure–activity relationships have become more complex to interpret and many existing ligands have yet to be evaluated at this subtype. A_3 evaluation is necessary in order to have a complete receptor binding profile for a particular ligand. The A_{2B} receptor is not well characterised in terms of structure–activity relationships, with just two major structure–activity studies appearing in the literature.[73,74] Although these studies were known to be at an A_2 receptor, it was not until several years later that the receptor was characterised as the A_{2B} subtype.[29] The recent cloning of the A_{2B} receptor should facilitate its future pharmacological study.

Adenosine receptor agonists

There exists no novel adenosine receptor agonist structures. All agonists are closely related in structure to the endogenous ligand, adenosine (**1**) (Figure 1). Structural and stereochemical requirements for the ribose moiety of adenosine agonists are strict. Alterations of either structure or stereochemistry result in a loss of receptor binding potency and possibly intrinsic activity. Recent studies have presented data for ribose modified adenosines at the A_1, A_{2A} and A_3 receptor subtypes, Table 5.[32,37,75] The ribose structure–activity relationships for the A_3 receptor have proven to be comparable

Table 5. Adenosine receptor binding affinity of ribose modified adenosine analogues, expressed as K_i in nM or % displacement at 10^{-4} M[32,37,75]

Compd	A_1K_i[a]	$A_{2A}K_i$[b]	A_3K_i[c]
β-L-Adenosine	29,000	25.4%	9.5%
α-D-Adenosine	350,000	128,000	14.2%
Adenine-β-D-arabino-furanoside	20.2%	26.0%	23.7%
2′-Deoxyadenosine	30.9%	38.9%	28.3%
3′-Deoxyadenosine	5.8%	26.3%	32.7%
5′-Deoxyadenosine	269	596	2830
2′-*O*-Methyladenosine	29.4%	49.0%	42.9%
3′-*O*-Methyladenosine	0.0%	8.0%	11.0%
MECA	83.6	66.8	72
NECA	6.3	10.3	113
Aristeromycin	3%	0.0%	200,000

[a]Displacement of specific [^{3}H]PIA or [^{3}H]CHA binding from rat brain membranes.

[b]Displacement of specific [^{3}H]CGS21680 or [^{3}H]NECA binding from rat striatal membranes.

[c]Displacement of specific [^{125}I]APNEA or [^{125}I]AB-MECA binding from membranes of CHO cells, stably transfected with the rat A_3-cDNA. Abbreviations: AB-MECA, N^6-(aminobenzyl)-5′-*N*-methylcarboxamidoadenosine; MECA, 5′-*N*-methylcarboxamidoadenosine.

to the much earlier characterised A_1 and A_{2A} receptors; this suggests that requirements for the ribose binding domain are similar in the three receptor subtypes.

Endogenous adenosine is the β-D-adenosine isomer. Both β-L-adenosine and α-D-adenosine exhibit virtually no affinity at adenosine A_1, A_{2A} or A_3 receptors. Inversion of the stereochemistry of the 2′-hydroxyl group (adenine-β-D-arabinofuranoside) gives rise to low receptor affinity. These results highlight the strict stereochemical requirements for the ribose moiety of adenosine. 2′-Deoxyadenosine, 3′-deoxyadenosine, 2′-*O*-methyladenosine and 3′-*O*-methyladenosine all have low receptor binding affinity. Modification of the 2′- or 3′-hydroxyls results in a greater loss of receptor binding affinity than modification of the 5′ hydroxyl. Substitution of the 5′-hydroxyl group of adenosine is better tolerated than substitution of the 2′- or 3′- hydroxyls. 5′-Deoxyadenosine has a K_i in the vicinity of 10^{-7} M at the A_1 and A_{2A} subtypes, and 10^{-6} M at A_3 subtype. 5′-Uronamide derivatives of adenosine possess good receptor affinity, with the 5′-*N*-ethyluronamide derivative (NECA) having greatest potency at the A_1 and A_{2A} subtypes, and the 5′-*N*-methyluronamide (MECA) derivative having greatest potency at the A_3 subtype.[32] The carbocyclic derivative, aristeromycin, has essentially no affinity at adenosine A_1 or A_{2A} subtypes, and weak affinity for the A_3 subtype, indicating the importance of the ring oxygen.

Few endocyclic modifications of the purine heterocycle of adenosine are tolerated, Table 6.[37,75] 3-Deazaadenosine is inactive, while 7-deazaadenosine has very low binding affinity at the rat A_1, A_{2A} and A_3 subtypes. The 1-deaza modification is much better tolerated, especially at the A_3 receptor. 1-Deaza-analogues of 2-CADO and NECA demonstrate that this endocyclic modification is dependent on the nature of simultaneous modifications at the C2 or the 5′-position. Other endocyclic modifications so far tested at only the A_1 and A_{2A} subtypes (human fibroblast cell line) include: 9-deaza, 8-aza, 7-deaza-8-aza, 8-aza-9-deaza and 2-aza analogues of adenosine.[73] All exhibit very low receptor binding affinity except for 2-azaadenosine which has binding affinity greater than that of adenosine.

Table 6. Adenosine receptor binding affinity of endocyclic modified adenosine analogues, expressed as K_i in nM or % displacement at 10^{-4} M[37,75]

Compd	A_1K_i[a]	$A_{2A}K_i$[b]	A_3K_i[c]
7-Deazaadenosine	21,500	59,800	61,700
3-Deazaadenosine	> 100,000	48.3%	38.9%
2-CADO	9.3	63	1890
1-Deaza-2-CADO	226	163	2480
NECA	6.3	10.3	113
1-Deaza-NECA	51	580	703

[a]Displacement of specific [^{3}H]PIA or [^{3}H]CHA binding from rat brain membranes.
[b]Displacement of specific [^{3}H]CGS21680 or [^{3}H]NECA binding from rat striatal membranes.
[c]Displacement of specific [^{125}I]APNEA or [^{125}I]AB-MECA binding from membranes of CHO cells, stably transfected with the rat A_3-cDNA.

Many N^6 substituted adenosine derivatives have been synthesised and evaluated for adenosine receptor binding affinity. The N^6 region at both the A_1 and A_{2A} subtypes has been mapped by virtue of the extensive structure–activity relationships available for these subtypes.[76,77] More recently, and consequently to a lesser extent, ligands have been evaluated at the A_3 subtype. Selected compounds, supporting a general discussion of the N^6 binding domain requirements of adenosine receptors, are presented in Table 7.[37] Modification at the N^6 position of adenosine is well tolerated, leading to potent agonists. With few exceptions substitution at N^6 enhances A_1 selectivity relative to the A_{2A} and A_3 subtypes, the benzyl substituent is equipotent for the A_1 and A_3 subtypes. A hydrogen at N^6 is a requirement for high affinity, with *N,N*-disubstituted derivatives exhibiting greatly reduced receptor binding affinity. Due to the large relative loss in affinity, this structure–activity relationship indicates that the N^6 hydrogen may be involved as proton donor in the formation of a hydrogen bond with the receptor protein. It is possible however, that this may also be indicative of an unfavourable steric interaction. The binding domain for the N^6 substituent is large and hydrophobic throughout all subtypes, tolerating both cycloalkyl and arylalkyl substituents. Finally the N^6 binding domain recognises

Table 7. Adenosine receptor binding affinity of N^6-exocyclic modified adenosine analogues, expressed as K_i in nM[37]

Compd	A_1K_i[a]	$A_{2A}K_i$[b]	A_3K_i[c]
N^6-(Methyl)adenosine	360	4,600	—
N^6-(Dimethyl)adenosine	10,000	28,900	32,500
N^6-(Cyclopentyl)adenosine	0.59	462	240
N^6-(Cyclohexyl)adenosine	1.3	514	167
N^6-(Phenyl)adenosine	4.62	663	802
N^6-(Benzyl)adenosine	120	285	120
N^6-(Phenethyl)adenosine	12.7	161	240
N^6-(*R*-Phenylisopropyl)adenosine	1.2	124	158
N^6-(*S*-Phenylisopropyl)adenosine	49.3	1,820	920

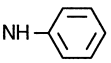
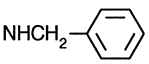
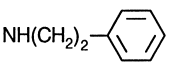
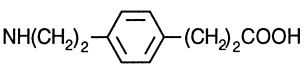
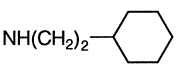
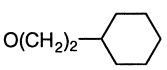
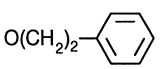
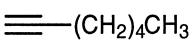
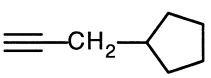
[a]Displacement of specific [^{3}H]PIA or [^{3}H]CHA binding from rat brain membranes.
[b]Displacement of specific [^{3}H]CGS21680 or [^{3}H]NECA binding from rat striatal membranes.
[c]Displacement of specific [^{125}I]APNEA or [^{125}I]AB-MECA binding from membranes of CHO cells, stably transfected with the rat A_3-cDNA.

stereoselectivity as evidenced for example by the superior potency of N^6-(*R*-phenylisopropyl)adenosine relative to N^6-(*S*-phenylisopropyl)adenosine.

Synthesis of C2-substituted adenosines is more difficult than of N^6-substituted adenosines, hence the structure–activity relationships for C2-substituted adenosines are not as extensively studied.[78] In general, C2 substitution leads to A_{2A} selective agonists, often as a result of lowering A_1 affinity and/or enhancing A_{2A} affinity. Few C2-substituted adenosines have been evaluated at the A_3 receptor, however both 2-CADO and 2-(phenylamino)adenosine have very poor affinity at the A_3 subtype relative to the A_1 and A_{2A} subtypes. The C2 domain of the A_{2A} receptor has been mapped.[78] This model was derived from structure–activity relationships for 2-alkoxyadenosines, and was later supported by 2-aralkoxyadenosines,[79] 2-aminoadenosines,[80,81] 2-(*N*′-alkylidenehydrazino)adenosines,[82] 2-(*N*′-aralkylidenehydrazino)adenosines,[83] 2-alkynyladenosines[84,85] and 2-(cycloalkylalkynyl)adenosines,[86] Table 8. A subdomain accommodates the atom linking the C2 substituent and the purine heterocycle. A methylene bridge acts as a spacer between the atom and a terminal hydrophobic subdomain, two methylenes being the optimum spacing. The hydrophobic subregion accommodates alkyl, cycloalkyl or aryl substituents. The cyclic hydrophobic groups may be further substituted leading to increased A_{2A} selectivity.

Potent and selective adenosine agonists, as described above, are the result of modifying the parent ligand adenosine by substitution, namely at N^6 or C2 of the purine heterocycle or the 5′ position of the ribose moiety. However it is a combination of substituents (i.e., multiply substituted adenosines) that lead to the most potent and subtype selective ligands, Table 9. The compounds in Table 9 are representative only, and many more examples exist. Interestingly, the structure–activity relationships are not always predictable based on the sum of effects of the singly substituted compounds, to the extent that an unfavourable singly substituted derivative does not preclude enhanced activity in a multiply substituted derivative. Such compounds highlight the importance of modifying more than one domain in producing adenosine derivatives with high subtype selectivity and receptor binding affinity.

Table 8. Adenosine receptor binding affinity of C2-exocyclic modified adenosine analogues, expressed as K_i in nM[37,80,85,86,114]

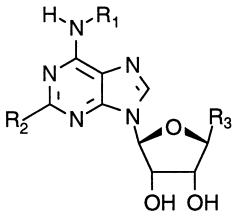
Compd	A_1K_i[a]	$A_{2A}K_i$[b]	A_3K_i[c]
Cl	9.3	63	1890
	560	119	4390
	10,471	5,888	—
	977	68	—
	12,589	74	—
	11,748	22	—
	1,600	22	—
	130	17	—
	28.4	2.8	—
	162	2.3	—

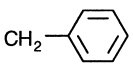
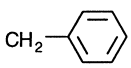
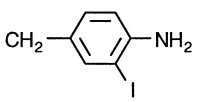
[a]Displacement of specific [^{3}H]PIA, [^{3}H]CHA or [^{3}H]CCPA binding from rat brain membranes.
[b]Displacement of specific [^{3}H]CGS21680 or [^{3}H]NECA binding from rat striatal membranes.
[c]Displacement of specific [^{125}I]APNEA or [^{125}I]AB-MECA binding from membranes of CHO cells, stably transfected with the rat A_3-cDNA.

Adenosine receptor antagonists

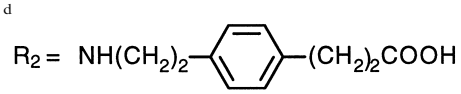
In contrast to agonists, adenosine receptor antagonists are novel in structure compared to adenosine. A comparison of different classes of antagonists demonstrates that, although diverse in structure, they do share some common structural features. In general the structures are: (i) planar; (ii) aromatic or π electron rich; and (iii) nitrogen-containing heterocycles.[2] There are exceptions to these generalisations, including benzo[*b*]furan[87] and tetrahydrobenzothiophenone[88] derivatives, with oxygen and sulfur containing heterocycles, respectively. The heterocycles are most often 6:5 fused bicyclics or 6:6:5 fused tricyclics, substituted with hydrophobic substituents. Additionally, antagonists lack the ribose moiety which is essential for agonist activity.

Table 9. Adenosine receptor binding affinity and selectivity of $N^6(R_1)$, C2(R_2), 5′(R_3)-substituted adenosine analogues, expressed as K_i in nM[32,37,80,81]



R_1	R_2	R_3	A_1[a]	A_{2A}[b]	A_3[c]	A_{2A}/A_1	A_3/A_1	A_{2A}/A_3
H	Cl	CH_2OH	9.3	63	1,890	6.7	203	
	H	CH_2OH	0.59	462	240	783	407	
	Cl	CH_2OH	0.6	950	237	1,583	395	
H	H	CONHEt	5.1	9.7	—	1.9		
H	See (d)	CH_2OH	12,589	74	—	0.0059		
H	See (d)	CONHEt	1,800	19	—	0.011		
H	H	CONHMe	83.6	66.8	72		0.86	0.93
	H	CH_2OH	120	285	120		1.0	2.4
	H	CONHMe	898	597	16		0.018	37
	H	CONHMe	18	197	1.3		0.072	160

[a]Displacement of specific [^{3}H]PIA or [^{3}H]CHA binding from rat brain membranes.
[b]Displacement of specific [^{3}H]CGS21680 or [^{3}H]NECA binding from rat striatal membranes.
[c]Displacement of specific [^{125}I]APNEA or [^{125}I]AB-MECA binding from membranes of CHO cells, stably transfected with the rat A_3-cDNA.
[d]



The first adenosine receptor antagonists reported were the naturally occurring xanthines caffeine and theophylline.[19] They exhibited weak affinity and subtype selectivity at adenosine receptors. A multitude of xanthines have since been synthesised in the quest for potent and selective ligands, and this class have now been optimised to the extent that both potent and selective A_1 and A_{2A} antagonists exist. More recently xanthines have been evaluated at the A_3 receptor.[33,37,89,90] A_3 affinity is dependent on species,[14] hence structure–activity relationships become more complex. A_3 species differences will be discussed separately.

As is the case for agonists, potent and selective xanthine antagonists stem from multiple substitutions of the parent heterocycle. Substitutions at N1, N3, N7 and C8 of xanthine (**2**) (Figure 1) contribute most to potency and selectivity at A_1 and A_{2A} receptors. The structure–activity

relationships for the N1 and N3 substituents of xanthines have been extensively studied at A_1 and A_{2A} receptors[91–93] and to a lesser extent at A_3 receptors.[33,37] Alkylation at both N1 and N3 of xanthines is required to maximise adenosine receptor binding affinity at both the A_1 and A_{2A} receptor subtypes, the rank order of potency at both subtypes is methyl < ethyl < *n*-propyl ≤ *iso*butyl,[92] Table 10. At the rat A_3 receptor 1,3-disubstituted xanthines exhibit poor affinity.[33,37] The N1, N3 structure–activity relationships are consistent for a variety of C8 substituted xanthines, a representative series with C8 as phenyl is presented in Table 10. At the A_1 receptor N1 and N3 as *n*-propyl are the optimum alkyl substituent combination, each *n*-propyl contributing equally to enhancement of binding compared to methyl at the same position. At the A_{2A} receptor the N3 alkyl group contributes markedly more to binding than the N1 alkyl group and, as for the A_1 receptor, N3 as *n*-propyl is preferred. At N1 there is little change in affinity by having *n*-propyl in place of methyl.

The most significant enhancements in affinity and subtype selectivity comes with substitution of the C8 position of xanthines. C8 substitution combined with N1 and N3 (and sometimes N7) substitution have led to the development of potent and selective A_1 and A_{2A} xanthines, Table 11. The C8 substituent exhibits stereoselectivity in a manner that parallels the N^6 substituent of agonists, as evidenced from the receptor binding affinity of 8-*RS*-, -*R*- and -*S*-phenylisopropylxanthine.[94] Adenosine A_1 potency and selectivity is readily gained by the introduction of large hydrophobic groups at C8.

Table 10. Adenosine receptor binding affinity of 1,3-disubstituted and 1,3,8-trisubstituted xanthines (**2**), expressed as K_i in nM or % displacement at 10^{-4} M[37,92,93]

R_1	R_3	R_8	A_1K_i[a]	$A_{2A}K_i$[b]	A_3K_i[c]
Methyl	Methyl	H	14,000	14,000	23.1%
Ethyl	Methyl	H	15,000	5,000	—
Ethyl	Ethyl	H	3,300	3,000	—
n-Propyl	*n*-Propyl	H	700	2,700	—
*iso*Butyl	*iso*Butyl	H	500	1,700	—
n-Butyl	*n*-Butyl	H	500[d]	29,300[e]	143,000
n-Propyl	*n*-Propyl	Cyclopentyl	0.23	230	18.7%
n-Propyl	*n*-Propyl	Phenyl	2.75	116	—
n-Propyl	Methyl	Phenyl	6.96	553	—
Methyl	*n*-Propyl	Phenyl	6.30	93.3	—
Methyl	Methyl	Phenyl	60	644	—

[a]Displacement of specific [^{3}H]CHA binding from rat brain membranes.
[b]Displacement of specific [^{3}H]NECA binding from rat striatal membranes.
[c]Displacement of specific [^{125}I]APNEA binding from membranes of CHO cells, stably transfected with the rat A_3-cDNA.
[d]Displacement of specific [^{3}H]PIA binding from rat brain membranes.
[e]Displacement of specific [^{3}H]CGS21680 binding from rat striatal membranes.

Table 11. Adenosine receptor binding affinity for 8-substituted xanthines (**2**), expressed as K_i in nM[21,29,92–95]

R_1	R_3	N_7	R_8	A_1K_i[a]	$A_{2A}K_i$[b]	A_{2a}/A_1
Methyl	Methyl	H	H	14,000	14,000	1
Methyl	Methyl	H	Phenyl	60	644	11
Methyl	Methyl	H	Cyclopentyl	11	1,440	130
Propyl	Propyl	H	Phenyl	2.75	116	42
Propyl	Propyl	H	Cyclopentyl	0.23	230	1,000
Propyl	Propyl	H	Cyclohexyl	0.30	320	1,067
Propyl	Propyl	H	3-Noradamantyl	1.3[c]	380	290
Propyl	Propyl	H	*R*-Phenylisopropyl	6.9	157	23
Propyl	Propyl	H	*S*-Phenylisopropyl	60.7	848	14
Propyl	Propyl	H	*RS*-Phenylisopropyl	32.6	644	20
Propyl	3-Aminophenethyl	H	Cyclopentyl	0.23	2,000	8,700
Methyl	Methyl	Methyl	3-Chlorostyryl	28,200	54	0.0019

[a]Displacement of specific [^{3}H]CHA binding from rat brain membranes unless otherwise indicated.
[b]Displacement of specific [^{3}H]NECA binding from rat striatal membranes.
[c]Displacement of specific [^{3}H]CHA binding from guinea pig forebrain membranes.

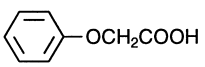
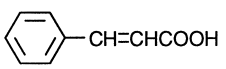
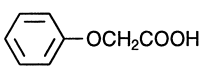
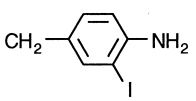
The structure–activity relationships demonstrate that C8-cycloalkyl substituents lead to very high A_1 affinity and subtype selectivity, with cycloalkyl preferred to phenyl substitution, Table 11.

Potent and selective A_{2A} xanthine antagonists were lacking until recent years. 8-Styryl derivatives of 1,3-dimethyl xanthines were found to be highly A_{2A} selective.[95] This study has been criticised on several accounts; firstly A_1 and A_{2A} receptor binding was measured in different species and secondly, insufficient Me_2SO was added to receptor binding assay experiments, possibly causing solubility problems.[96] A large series of 8-styryl xanthines has been prepared and evaluated for rat A_1 and A_{2A} receptor binding affinity.[96] The structure–activity relationships demonstrated: (i) the substitution pattern of the styryl ring is determinant of potency and selectivity, with *meta* substitution most favoured; (ii) larger alkyl groups at N1 and N3 increased affinity for both A_1 and A_{2A} receptors, with an overall loss in A_{2A} selectivity; (iii) substitution of the N7 hydrogen for methyl improved A_{2A} selectivity, and (iv) A_{2A} selectivity and moderate potency was maintained with long chain extension at the *para* position of the styryl moiety. Photolability of the 8-styrylcaffeines is a drawback.[97]

Species differences in A_3 receptor binding affinity of xanthine antagonists are significant.[14,33,37,89,90,98] The relatively low amino acid sequence homology (Table 2) exhibited between cloned sheep and human A_3 receptors compared to cloned rat A_3 receptor is most likely instrumental in these pronounced pharmacological differences. Xanthines have weak affinity for rat A_3 receptors, however a small number of xanthines exhibit high affinity at both sheep and human A_3 receptors, representative compounds are presented in Table 12. C8 substitution enhances sheep and human receptor affinity, while the rat receptor shows limited improvement with respect to this substitution. 8-Phenylxanthines with *para*-acidic substituents exhibit greatest affinity at sheep and human receptors. Differences between sheep and human are apparent, most prominently for the 8-cyclopentyl substituent which is 65-fold more potent at the human receptor.

Numerous structurally diverse non-xanthine adenosine antagonists have been identified.[2] Unlike xanthines the structure–activity relationships for these novel classes of antagonists are not well defined, nor have they been optimised to achieve maximal adenosine receptor binding affinity or subtype selectivity. Those that are selective are generally A_1 selective, with few A_{2A} selective ligands. The search for A_3 selective compounds has been approached in two ways. The first is to evaluate existing adenosine antagonists, due to species differences it is important to evaluate such compounds at the human A_3

Table 12. Species differences in adenosine A_3 receptor binding affinity of xanthines (**2**), expressed as K_i in nM or % displacement at 10^{-4} M. $R_1 = n$-Propyl unless otherwise indicated[33,37,89,90]

Compd	R_8	R_3	Rat	Sheep[c]	Human[d]
Theophylline	H	$R_1 = R_3 = CH_3$	23.1%[a]	424,000	—
DPCPX		$CH_2CH_2CH_3$	18.7%[a]	49,300	760
XCC		$CH_2CH_2CH_3$	75,700[b]	—	—
BWA1433		$CH_2CH_2CH_3$	15,000[b]	21	55
BWA522			1,170[b]	3	18

[a]Displacement of specific [^{125}I]APNEA binding from membranes of CHO cells, stably transfected with the rat A_3-cDNA.
[b]Displacement of specific [^{125}I]AB-MECA binding from membranes of CHO cells, stably transfected with the rat A_3-cDNA.
[c]Displacement of specific [^{125}I]ABA binding from membranes of CHO cells, stably transfected with the sheep A_3-cDNA.
[d]Displacement of specific [^{125}I]ABA binding from membranes of CHO cells, stably transfected with the human A_3-cDNA.
Abbreviations: XCC, 8-[4-[[[carboxy]methyl]oxy]phenyl]-1,3-dipropylxanthine; BWA1433, 1,3-dipropyl-8-[4-(carboxyethynyl) phenyl]xanthine.

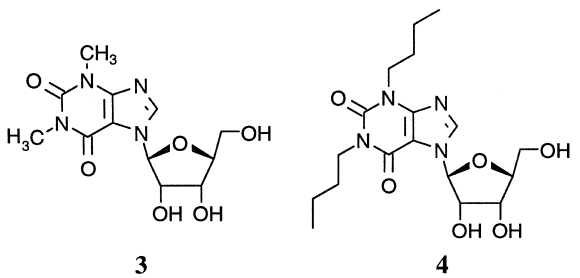
receptor. The triazoloquinazolines, which bind poorly to rat A_3 receptors, were discovered by this approach.[99] This class of compounds have been developed to give the first potent and selective human A_3 antagonists. Many existing classes of compounds still remain to be evaluated at the human A_3 receptor. The second approach is to screen compound libraries. The structurally novel flavanoids and dihydropyridines have been identified by this approach, their development into A_3 selective compounds has followed.[100,101]

Adenosine receptor partial agonists

A partial agonist is a compound whose intrinsic activity is less than that of a full agonist. There are several potential advantages to the use of adenosine receptor partial agonists compared to full agonists: (i) the cardiovascular hypotensive effects caused by adenosine agonists is a limiting factor to their potential therapeutic use, partial agonists may circumvent this deleterious side effect; (ii) partial agonists may induce less receptor downregulation and desensitisation; and (iii) partial agonists may be more subtype selective.[102,103]

Removal of the ribose moiety of adenosine agonists abolishes intrinsic activity, these compounds are therefore antagonists.[104–106] The removal of the 2′-hydroxyl or 3′-hydroxyl groups from potent N^6-substituted adenosine agonists (i.e., deoxy derivatives) not only lowered affinity, but also intrinsic activity, such compounds are therefore partial agonists.[103]

Theophylline-7-riboside (**3**) was one of the first adenosine A_1 receptor partial agonists identified.[102] 1,3-Dibutylxanthine-7-riboside (**4**) was shown to be a partial agonist at the adenosine A_3 receptor.[37] These compounds consist of a hybrid agonist:antagonist (i.e., ribose:xanthine) structure, Figure 5. 1,3-Dibutylxanthine-7-riboside-5′-*N*-methylcarboxamide, also a hybrid structure but including the A_3 agonist enhancing 5′-methyl uronamide substituent, is an A_3 selective full agonist.[35] These hybrid compounds are interesting, having allowed the conversion of a full antagonist to a partial agonist to a full agonist.



3 4

Figure 5. Adenosine receptor partial agonists, consisting of the hybrid agonist:antagonist (ribose:xanthine) structure. Theophylline-7-riboside (**3**)[102] and 1,3-dibutylxanthine-7-riboside (**4**).[21]

Molecular Modelling

Molecular modelling of the adenosine receptor binding site has been approached from two alternate fronts. The first approach was to model the adenosine receptor ligands (small molecule modelling), while the second was to model the actual adenosine receptor proteins (large molecule modelling).

Several models of the adenosine receptor pharmacophore using ligand based computer modelling techniques have been presented in the literature.[94,107–109] The aim of these molecular modelling studies was to develop a pharmacophore which would direct the future design of potent and selective ligands, leading to rational rather than random discovery. The models are based on rationalising the structure–activity data for a range of adenosine agonists and antagonists.

Prior to discussion of the individual models it is worthwhile to consider that each share some common concepts in relation to their development. Firstly, superimposition of various ligands in the development of each of the models was based on maximising ligand-receptor binding interactions. These interactions encompass steric, electrostatic and hydrophobic complementarity.[107] In addition to these, the importance of correlating hydrogen bond donors has been realised.[94,109] Each model assumed that adenosine agonists and antagonists bind to a common binding site on the receptor.[110] This facilitated the direct modelling of agonists against antagonists.

A brief summary of the extent of knowledge of adenosine research at the time the models were published is also worthwhile in order to place in perspective the significance of their contribution to adenosine research. Potent and selective A_1 agonists and antagonists, and potent and selective A_{2A} agonists were known, however there was a lack of selective A_{2A} antagonists, the A_3 receptor was not yet discovered or characterised, and molecular cloning of receptor subtypes was in its infancy, so there was limited structural knowledge of the receptors. Consequently the modelling studies focussed on the A_1 receptor, due primarily to the availability of potent and selective A_1 agonists and antagonists. Only one of the models addressed the A_{2A} receptor, in terms of A_1/A_{2A} selectivity.[109] It is unfortunate that little attention has been focussed on either refining or expanding these models to encompass A_{2A} selective xanthine antagonists, and A_3 receptor agonists and antagonists, now that they exist.

In development of the 'flipped' model, initial superimposition of adenosine with theophylline, the prototypic xanthine antagonist, by superimposition of the purine heterocycles (the most obvious manner of superimposition) was undertaken.[107] Although only the starting point for the development of the 'flipped' model, this superimposition has been designated a model in its own right, namely the 'standard' model, Figure 6a.

The 'standard' model has the atoms N1, N3, N7 and N9 of both ligands coinciding, and possesses good steric, hydrophobic and electrostatic overlap. However, if an A_1 selective N^6-substituted agonist is modelled with an A_1 selective C8-substituted xanthine the exocyclic N^6, C8 substituents occupy different spatial regions and do not enhance steric, hydrophobic or electrostatic overlap, Figure 6b.[111] An alternative superimposition of the heterocycles is shown in Figure 6c. N1, N3, N7 and N9 of adenosine coincides with C2, C6, N9 and N7, respectively of the xanthine. This orientation maintains the steric and hydrophobic overlap of the 'standard' model, however considerably improves the electrostatic overlap. Due to the 180° rotation of the xanthine about its long axis (relative to the xanthine orientation in the 'standard' model) this orientation was named the 'flipped' model. Again, if both N^6- and C8-substituted compounds are modelled, steric and hydrophobic overlap is not improved, Figure 6d.[111] Support for the flipped orientation over the standard orientation is evidenced from the A_1 receptor affinity of theophylline-7-ribonucleosides and lack of affinity of theophylline-9-ribonucleosides.[112,113] Essentially the 'flipped' model proposes four discrete binding domains in the A_1 receptor binding site: (i) a purine binding domain, common to agonists and antagonists; (ii) a ribose binding domain, for agonists; (iii) an N^6 binding domain, for A_1 selective agonists; and (iv) a C8 binding domain, for A_1 selective xanthines.

The 'N^6-C8' is a model based on the hypothesis that the C8 substituent of xanthines binds to the same region of the receptor as the N^6 substituent of adenosine derivatives (i.e., that these binding domains are not discrete was proposed).[29,94] Xanthines were suggested to bind 'backwards' compared to adenosine, with the five-membered ring of theophylline corresponding roughly in position to the six-membered ring of adenosine, based on the A_1 selectivity conferred by a cyclopentyl group in N^6-cyclopentyladenosine and 8-cyclopentyltheophylline.[29] This hypothesis was elaborated and supported both by structure–activity relationships for existing ligands and for 8-*RS*-, -*R*- and -*S*-phenylisopropylxanthine, lending support to this model.[94] The 'N^6-C8' model is shown in Figure 6e. N^6, N1, N3 and N9 of the agonist approaches N9, N7, N1 and N3 of the xanthine. This model possesses good, steric, electrostatic and hydrophobic overlap, and places potential hydrogen bonding sites in close proximity, namely the N^6-hydrogen and N7-hydrogen of the agonist with the N7-hydrogen and O^6 of the xanthine. The 'standard', 'flipped' and 'N^6-C8' models have been compared in terms of steric, electrostatic and hydrophobic overlap, as well as correlation of potential hydrogen bonding groups.[111] Very little difference was observed in overlap with the modelling of adenosine against theophylline, however when the more potent and A_1 selective N^6- and C8-substituted analogues were modelled, significant differences in overlap were observed, Figure 6b, d and e. They concluded that the 'N^6-C8' model was the most probable model based on the greatest overall steric and hydrophobic overlap, as well as placing potential hydrogen bonding functionalities in close proximity.

The 'three binding domain' model assesses A_{2A} selective C2-substituted adenosine agonists as well as A_1 selective N^6-substituted adenosine agonists and A_1 selective C8-substituted xanthines.[108,109] Ligands were superimposed on the basis that the C2, N^6 and C8 substituents bind to the same region of the A_1 and A_{2A} receptor, called the hydrophobic binding domain (Figure 6f and g). As for the 'N^6-C8' model, this model hypothesised that the N^6 and C8 substituents did not bind to discrete regions of the receptor but rather to a common region. The 'three binding domain' has been described as a 'C2-N^6-C8' model.[114] The model possesses good steric, electrostatic and hydrophobic overlap, and similarly to the 'N^6-C8' model places potential hydrogen bonding functionalities in close proximity, namely the N^6-hydrogen of the A_1 agonist with the C2-hydrogen of the A_{2A} agonist and the N9-hydrogen of the xanthine. For comparison with the other models, the N^6, N1 and N3 of the A_1 agonist coincides with N9, N3 and N1 of the A_1 xanthine antagonist. In addition to the hydrophobic binding domain the 'three binding domain' model also proposes a central aromatic binding domain and a ribose binding domain in the A_1 and A_{2A} receptors, Figure 7.

Molecular modelling of the receptor and of the receptor–ligand complex has been performed for the A_1, A_{2A} and A_3 receptor subtypes.[37,115–117] The aim of modelling from the receptor perspective is to identify potential interactions between the receptors amino acids and the bound ligand utilising docking experiments. If accurate, receptor based models should enhance the successful design of receptor subtype selective ligands by providing an understanding of the molecular basis of receptor-ligand recognition.

Detailed three-dimensional structures (from X-ray or NMR studies) for any of the 300 cloned and sequenced G protein-coupled receptors is nonexistent. Adenosine receptor modelling was therefore based on assumed structural homology with the structurally well defined

Fig. 6. Superimposition of ligands. The 'standard' model[107] (a) adenosine (red) and theophylline (blue); (b) with N^6- and C8-substituted ligands, CPA (red) and DPCPX (blue). The 'flipped' model[107] (c) adenosine (red) and theophylline (blue); (d) with N^6- and C8-substituted ligands, CPA (red) and DPCPX (blue). The 'N^6-C8' model[94], (e) with N^6- and C8-substituted ligands, CPA (red) and DPCPX (blue). The 'three binding domain' model[33] or 'C2-N^6-C8' model with C2-, N^6-, and C8-substituted ligands. (f) A_1 Agonist/A_1 antagonist: CPA (red) and DPCPX (blue); (g) A_1 agonist/A_{2A} agonist: N^6-*R*-PIA (red) and CGS21680 (blue). (h) A_1 Agonist/A_{2A} agonist/A_1 antagonist: N^6-*R*-PIA (red), CGS21680 (blue) and DPCPX (magenta).

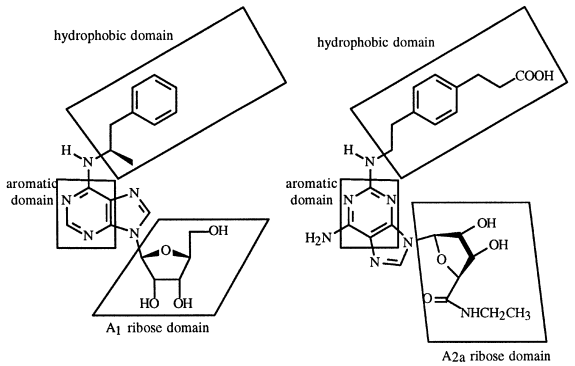


Figure 7. Hydrophobic, aromatic and ribose binding domains as proposed by the 'three binding domain' model.[33] (a) A_1 Receptor with N^6-*R*-PIA; (b) A_{2A} receptor with CGS21680.

protein, bacteriorhodopsin. This assumption is accepted despite essentially no amino acid sequence homology between bacteriorhodopsin and adenosine receptors. The structure of bacteriorhodopsin has been resolved to 3.5 Å using high-resolution electron cryo-microscopy.[118] The protein consists of seven alpha helical transmembrane spanning domains, which are distinguishable from the intra- and extracellular loops.

The atomic coordinates of the seven transmembrane domains of bacteriorhodopsin directed modelling of the adenosine receptors, with methodology similar to that described for other G protein-coupled receptors.[119] Initial structures were generated by 'mutation' of the bacteriorhodopsin amino acid sequence to the corresponding adenosine receptor amino acid sequence. This

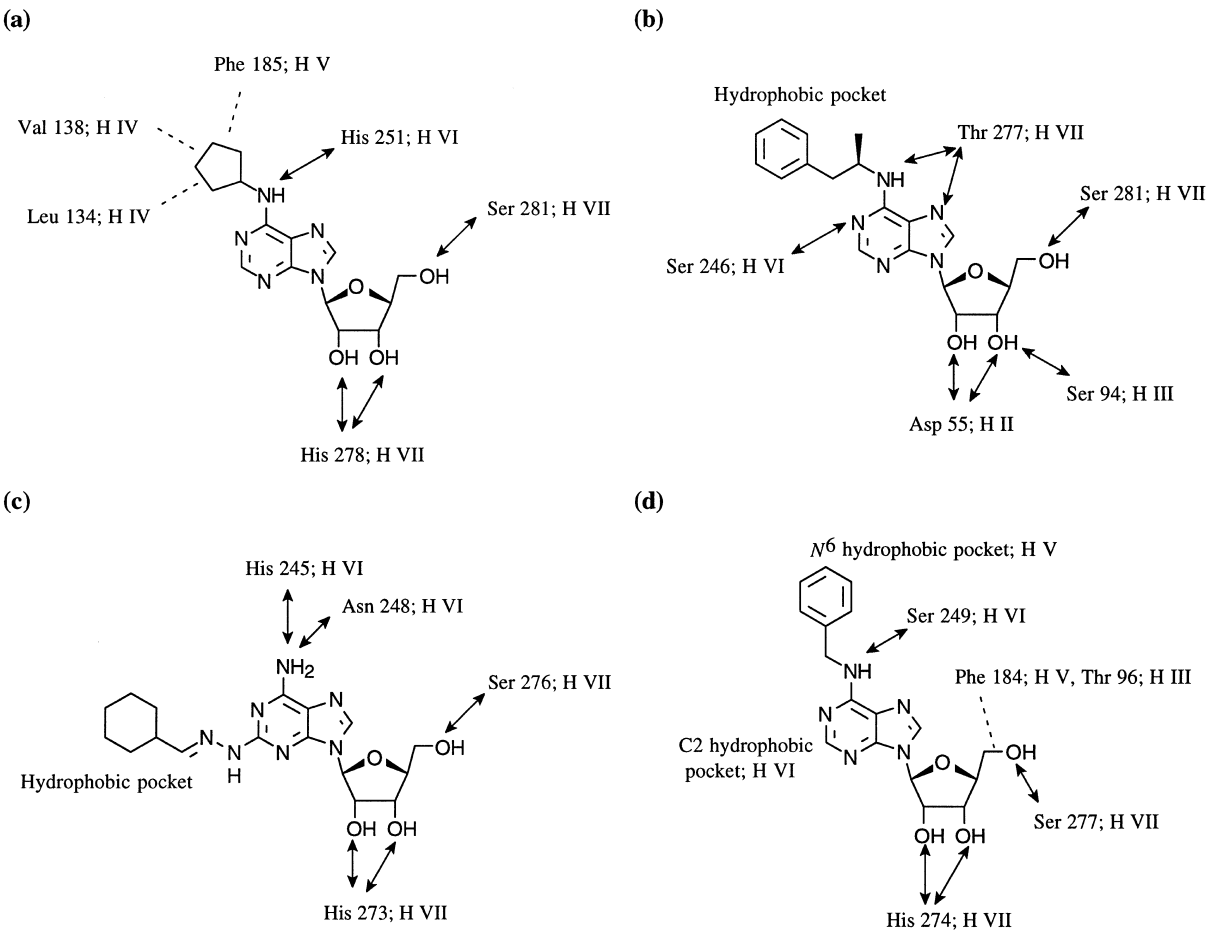


Figure 8. The ligand binding site model for the adenosine receptor subtypes. The models are based on docking of agonists into the receptor cavity to maximise interactions between the bound agonist and receptor amino acids. Double headed arrows represent potential hydrogen bonding interactions, while dashed lines represent potential hydrophobic interactions. (H = helix) (a) Canine A_1 receptor, modelled with N^6-(cyclopentyl)adenosine.[115] (b) A_1 Receptor, modelled with N^6-(*R*-phenylisopropyl)adenosine.[116] (c) Rat A_{2A} receptor modelled with 2-(cyclohexyl-methylidinehydrazino)adenosine, (SHA 174).[117] (d) Rat A_3 receptor modelled with N^6-(benzyl)adenosine.[37]

structure was then optimised by rotation of helices, energy minimisation, molecular dynamics and the limited knowledge available from biochemical studies. The final result resembled the molecular architecture of bacteriorhodopsin (i.e., the arrangement of the seven helices formed a funnel like cavity with the diameter of the extracellular side being larger than that of the intracellular side). The ligand binding site was deduced from docking potent and selective ligands (both agonists and antagonists) into the cavity formed by the receptor architecture. The docking strategy is highly speculative, with limited experimental evidence available to guide it (two significantly different models have been proposed for the A_1 receptor ligand binding site[115,116]). Agonists were studied first with antagonists then docked in accordance with the 'N^6-C8' superimposition, described earlier.[94] This strategy aimed to maximise favourable ligand-receptor interactions, i.e. to maximise steric, hydrophobic, electrostatic and hydrogen bonding complementarity between the bound ligand and receptor cavity. The results for the A_1 receptor are summarised in Figure 8a and b, for the A_{2A} receptor in Figure 8c and for the A_3 receptor in Figure 8d. These figures illustrate the points of interaction between the agonists studied and the receptor protein.

These models are not claimed to be definitive, but rather to serve as useful starting points to direct future studies aimed at model refinement. These studies include chemical modification and site-directed mutagenesis of the receptor proteins, and ligand synthesis to probe further structure–activity relationships. Kim et al.[120] recently published results of site-directed mutagenesis experiments, these experiments were guided by the A_{2A} model described above. Their results led them to postulate another A_{2A} ligand binding site model, Figure 9. The adenosine receptor sequence was modelled on the electron density map of rhodopsin, rather than bacteriorhodopsin. Rhodopsin, unlike bacteriorhodopsin, is a G protein-coupled receptor and the first of the cloned G protein-coupled receptors for which a projection map showing the configuration of the helices is available.[121] Although the model obtained is similar to the earlier A_{2A} ligand binding site model, some specifically different interactions are proposed.

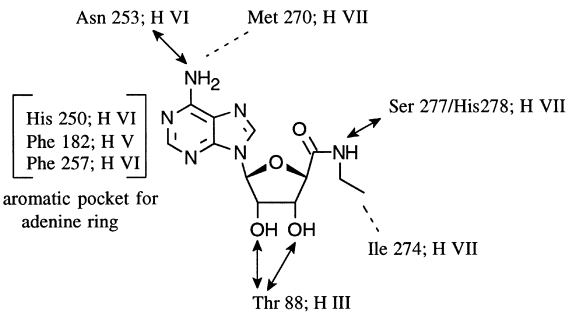


Figure 9. Human A_{2A} ligand binding site model, based on the structure of the G protein-coupled receptor rhodopsin, docked with 5′-*N*-ethylcarboxamidoadenosine. Double headed arrows represent potential hydrogen bonding interactions, while dashed lines represent potential hydrophobic interactions.[120]

Rapid advances in modelling studies from the receptor perspective are anticipated as a consequence of the support from molecular biology techniques, allowing testing and refinement of existing models. Identification of individual amino acids and domains involved in ligand binding is possible by mutagenesis studies, which take advantage of the ability to clone and express the adenosine receptor subtypes.[12,13] Mutagenesis experiments for the A_1, A_{2A} and A_3 subtypes have been described,[120] as well as the generation and study of chimeric A_1-A_3 receptors[122] and chimeric A_1-A_{2A} receptors.[123] Photoaffinity radiolabelling followed by proteolysis and amino acid sequencing of the fragments identified ligand binding to particular transmembrane regions of the A_{2A}AR.[124,125] The results to date have identified important regions of the receptor involved in ligand binding, and have demonstrated considerable overlap in the agonist and antagonist binding sites. Similar studies have not yet been performed for the A_{2B} receptor subtype.

Conclusion

The availability of cloned human adenosine receptors will increase the likelihood of developing selective agents for human application. Differences have been elucidated between the subtypes and increased structural knowledge of the adenosine receptors may allow more selective compounds to be developed. This is most apparent for the adenosine A_3 receptor in which case it is now clear that information developed on other than cloned human A_3 should be de-emphasized. In addition, the specific tissue localization observed for the adenosine A_3 receptor may overcome what has been one of the largest barriers to development of adenosine based therapeutics.

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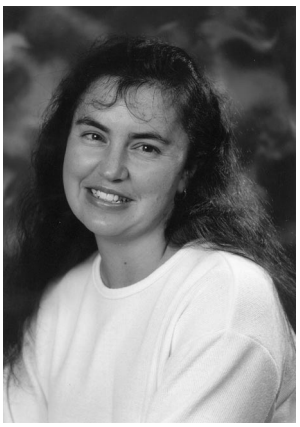
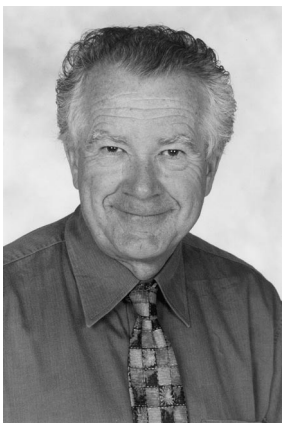
Bibliographical Sketch



Sally-Ann Poulsen was born in New South Wales, Australia in 1968. She received her BSc and BSc (Hons) degrees from Griffith University, Brisbane, Australia. Her Ph.D. studies were carried out under the supervision of Professor Ronald J. Quinn (Griffith University) and the Ph.D. degree awarded in 1996. She undertook postdoctorial training with Professor David J. Craik (University of Queensland) from July–December 1996 and is currently a Postdoctoral Fellow at Astra Charnwood (Astra Pharmaceuticals Ltd.), UK. As a student, Dr Poulsen was awarded several prizes for academic achievement, including a University Medal. Dr Poulsen's current research interests are in the field of Medicinal Chemistry. With a background in both synthetic organic chemistry and NMR she hopes to apply these skills to study structure–function relationships and molecular recognition of biologically important molecules.



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