Adenosine Receptors:
The Contributions by John W. Daly

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Abstract – John Daly played an important role in defining adenosine receptors as an important target for drug discovery. His systematic work characterized the effects of adenosine analogues on cyclic AMP in the brain that were antagonized by methylxanthines. He also played a decisive role in establishing these receptors as *bona fide* biochemical entities and contributed to the discovery of receptor heterogeneity. This brief review will cover some of his important early discoveries in the pharmacology and medicinal chemistry of adenosine receptors.

INTRODUCTION: INITIAL EVIDENCE FOR ADENOSINE RECEPTORS

The presence of receptor(s) for adenosine at which theophylline acted as an antagonist were postulated on the basis of functional data on atrial muscle.[1] However, it was predominantly the contributions by Theodore (Ted) Rall at the University of Virginia and John W. Daly at NIH that firmly established the concept of adenosine receptors. Rall was part of the original team that discovered 3′,5′-cyclic adenosine monophosphate (cyclic AMP) as the first example of a second messenger in 1957. Both Ted Rall´s and John Daly´s groups were interested in the regulation of neurotransmission in the brain and decided to use changes in intracellular cyclic AMP as a monitor of the actions of transmitters and modulators. Rall and his coworkers determined total levels of endogenous cyclic AMP in brain slices from several brain areas and species and detected stimulation by several different potential transmitter substances and by electrical stimulation.[2-5] John Daly and his group instead developed an alternate technique, radioactive prelabelling, in 1969.[6] They incubated the brain slices (again from different species and regions) with tritium-labeled adenine, which was incorporated into the ATP pool in the slices. In a second step, they could examine the conversion of the radiolabeled ATP into labeled cyclic AMP. This method was very thoroughly characterized and validated[7-10] and proved to be extremely versatile and sensitive. The method also allowed the study of release of adenosine from brain slices.[11, 12]

One of the most efficient stimulators of cyclic AMP accumulation in brain slices proved to be adenosine.[5,8-10] These effects were antagonized by theophylline (and other methylxanthines, Figure 1) but were enhanced by dipyridamole and papaverine, phosphodiesterase inhibitors that also prevent adenosine uptake into cells and thereby increase effective adenosine concentrations extracellularly.[5, 13] Furthermore, within a series of adenosine analogues, those compounds having an intact purine ring could mimic the effect of adenosine.[14] These data provided strong evidence for the presence of adenosine receptors on the cell surface at which methylxanthines acted as antagonists.

These data also contained important leads for the subsequent discovery of potent agonists and antagonists of adenosine receptors. John Daly and colleagues were among the first to systematically probe the effects of substitution on biological activity in the adenosine agonist (nucleosides) and antagonist (alkylxanthines) series. Adenosine and xanthine derivatives were synthesized with alterations at all of the amenable sites on the pharmacophore structures. Thus, by preparing series of analogues they established the fundamental structure activity relationships (SARs) for these biologically important receptors.

ADENOSINE IS A TRANSMITTER/MODULATOR IN BRAIN

A variety of depolarizing agents (e.g., ouabain, batrachotoxin, veratridine, and K^+) could increase cyclic AMP accumulation in brain slices.[11, 14, 15] Since the same stimuli actually released adenosine from the slices, and the cyclic AMP increase was reduced by the adenosine antagonist theophylline, it was reasonable to assume that "released adenosine was responsible for the enhanced accumulation of cyclic AMP elicited by depolarizing agents and electrical pulsation".[14] This conclusion was strengthened by the findings that methylxanthines did not reduce adenosine release and that adenosine deaminase reduced the cyclic AMP effect. Adenosine levels in slices increased by the same stimuli that caused release of adenosine, and this suggested that adenosine might not be released as a classical transmitter, but rather is formed on demand.

A large number of studies, comprehensively summarized in Daly´s monograph,[14] showed that adenosine was not the only factor responsible, but that it could actually interact with other types of agents. In particular it was shown that activation of α_1 adrenoceptors or H_1 histamine receptors could cause accumulation of cyclic AMP, if, and only if, adenosine was present. A full explanation for this facilitation may not be at hand even now, but a partial explanation appears to be that receptor-mediated activation of protein kinase C and elevation of intracellular calcium ion concentration can enhance ongoing receptor-mediated stimulation of cyclic AMP formation.[16-18]

METHYL XANTHINES AS ADENOSINE RECEPTOR ANTAGONISTS AND THEIR OTHER EFFECTS

Despite the fact that it was clear that methylxanthines such as theophylline (and caffeine) could act at micromolar concentrations as antagonists of adenosine actions, presumably by blocking putative adenosine receptors, this information was largely ignored, and these drugs were widely used as inhibitors of phosphodiesterase. Their biological effects were simplistically interpreted as an indication of a role of cyclic AMP. It became obvious that the two properties are not necessarily linked, since there were phosphodiesterase inhibitors that did not antagonize adenosine actions.[19] Not even in the xanthine family were the two properties necessarily linked as shown in a particularly important contribution that came from the combined efforts of the laboratories of Daly and Wells.[20] A key result from that paper is shown in Table 1. Of the compounds studied, 8-phenyltheophylline became a prototype for the development of more potent and selective adenosine receptor antagonists. Conversely, 7-benzyl-IBMX (Figure 1) was much more potent as an inhibitor of phosphodiesterases than adenosine receptors. This provided a guide for the early SAR studies of xanthines as adenosine antagonists: variable substitution, particularly small hydrophobic groups, at the 1 and 3 positions is much better tolerated than substitution at the 7 position. Homologation of the 1,3-dialkyl groups beyond methyl tended to enhance adenosine receptor antagonist potency.

theophylline

caffeine

7-benzyl-3-isobutyl-1-methylxanthine
(7-benzyl-IBMX)

R = Me, 8-phenyltheophylline
R = Et, 1,3-diethyl-8-phenylxanthine
(DPX)

3,7-dimethyl-1-propargyl-
xanthine (DMPX)

3-propylxanthine
(enprofylline)

xanthine amine congener, XAC

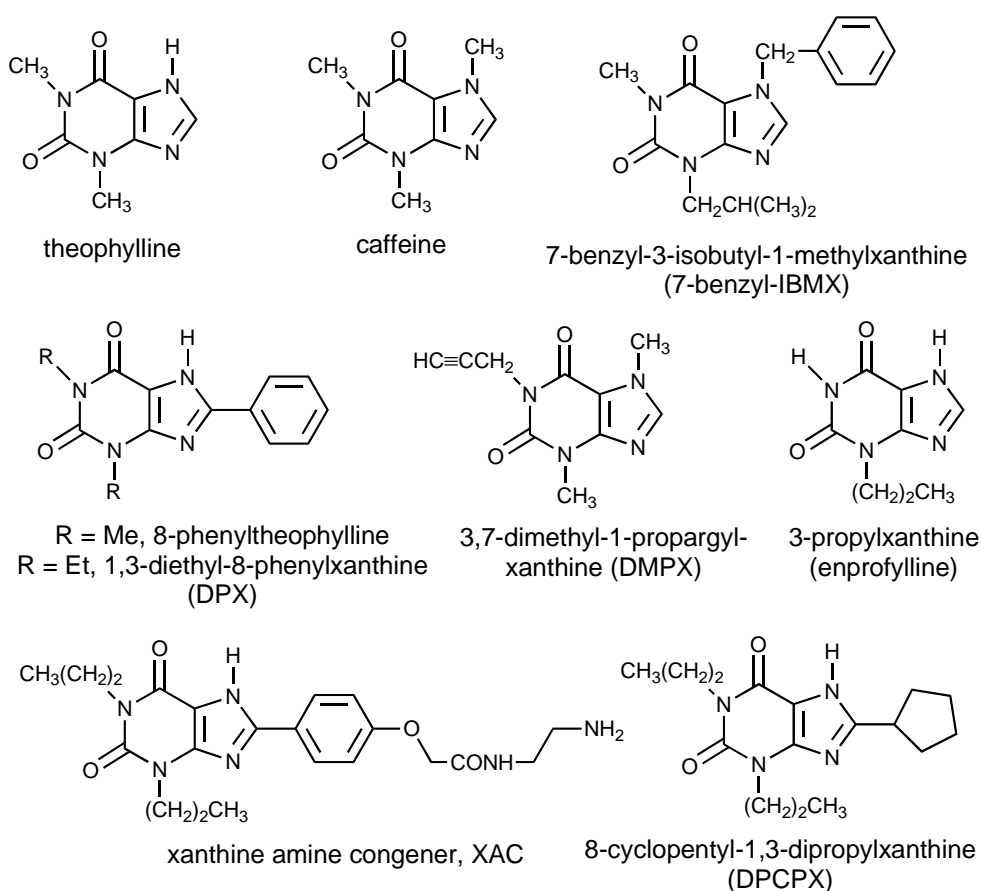8-cyclopentyl-1,3-dipropylxanthine
(DPCPX)

Figure 1. Structures of prototypical alkylxanthines studied as adenosine receptor antagonists and as inhibitors of phosphodiesterases.

Table 1. Potency of selected alkylxanthines as adenosine antagonists and phosphodiesterase inhibitors. Data from Smellie, Davies, Daly and Wells.[20]

Xanthine	IC50 mM			Ratio	Ratio
	ADO receptor	PDE Ca indep	PDE Ca dep	Ado antag/ inhib Ca indepPDE	Ado antag/ inhib Ca depPDE
3-Isobutyl-1-methyl xanthine (IBMX)	60	7.5	40	8	1.5
Theophylline	60	950	500	0.06	0.12
8-phenyl-theophylline	6	>100*	>100*	<0.06	<0.06
1,3-dibutyl xanthine	30	100	>100*	0.3	<0.3
7-benzyl IBMX	100	1.5	100	66	1
1-isoamyl-3-isobutyl xanthine	>>200*	100	40	>>2	>>5

*- poor solubility prevents accurate potency determination

The fact that methylxanthines act as adenosine antagonists has obvious implications for the interpretation of the effects of the most widely used of all psychoactive drugs, caffeine. Although it is somewhat weaker as an adenosine antagonist than theophylline, it is consumed in rather high doses. It could be calculated that the amounts consumed habitually by humans might be sufficient to cause blockade of adenosine receptors *in vivo* (Figure 2). Other potential biochemical targets of caffeine, such as Ca^{2+} release from the sarcoplasmic reticulum in muscle, appear to require higher doses. The findings noted above that endogenous adenosine appeared to modulate brain function raised the possibility that a pure adenosine antagonist could have its own biological actions, such as a CNS stimulant or a cognitive enhancer.[21-25] Nevertheless, it appears clear that caffeine, as well as caffeine analogues, act also on targets other than adenosine receptors. Alkylxanthine derivatives remain an interesting class of potential drugs that display a spectrum of activities and have typically low toxicity.[21] John Daly's exploration of the biological actions of caffeine (and other adenosine receptor ligands) and their relation to other neurotransmitter systems were not limited to model *in vitro* systems but included *in vivo* studies in behavioral models.[23]
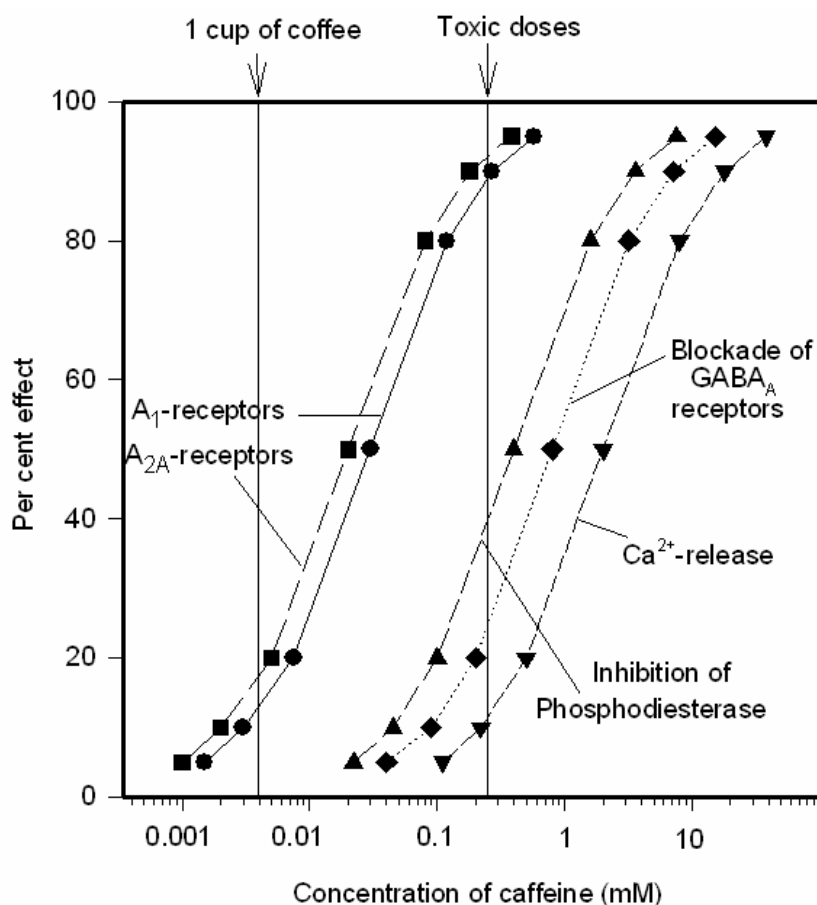
Figure 2. The parallel dose response curve for various actions of caffeine that occur at different concentrations. The concentrations in the body following dietary caffeine intake correspond most closely to those relevant to adenosine receptor antagonism (from Daly and Fredholm[24]).

BINDING STUDIES

Despite the biochemical evidence for adenosine receptors from the second messenger effects detailed above, more compelling evidence was needed to convince the majority of the scientific community. A first step would be detect the receptors using a radioligand binding assay. John Daly established a collaboration with Solomon Snyder at Johns Hopkins University, and Robert Frederick (Fred) Bruns acted as a postdoctoral liaison between the laboratories around 1980. Their efforts involved the establishment of one high afffinity agonist radioligand of what was to be termed the A_1 adenosine receptor, [³H]N^6-cyclohexyladenosine ([³H]CHA, structure in Figure 4), and one antagonist radioligand [³H]1,3-diethyl-8-phenylxanthine ([³H]DPX, structure in Figure 1) as demonstrated in the adenosine receptor saturation curves shown in Figure 3.[26]
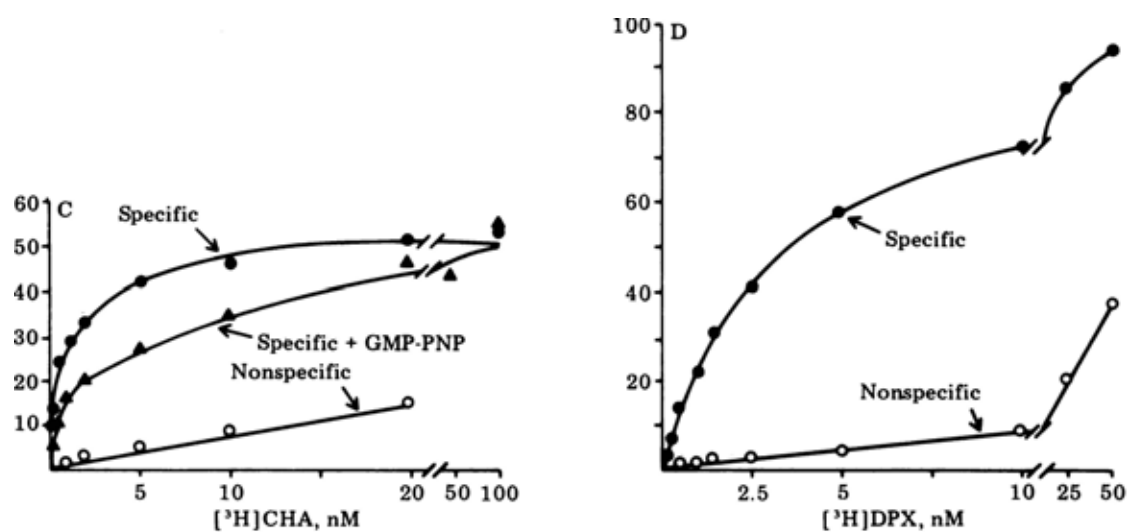
Figure 3. Saturation binding of an agonist, [^{3}H]CHA (left panel), and an antagonist, [^{3}H]DPX (right panel), to membranes from bovine brain (from Bruns *et al.*[26] by permission). The agonist binding could be modulated by guanine nucleotides, such as GMP-PNP, as expected for a G protein-coupled receptor.

It was known that valid radioligand assays required affinities to be in the low nanomolar range, and therefore, a novel antagonist had to be developed. This first antagonist for adenosine receptors, [^{3}H]DPX, satisfied the affinity requirement and initially became a standard tool. However, the hydrophobicity of DPX was a limitation, and a related 8-phenylxanthine with more favorable physicochemical properties and higher affinity, [^{3}H]XAC, supplanted the use of [^{3}H]DPX.[38] Soon thereafter, another key antagonist probe for characterization of adenosine receptors DPCPX (Figure 1) was discovered independently in Daly's lab[44] and by Bruns,[45] at that time working in the pharmaceutical industry.

At almost the same time two more radioligand binding studies demonstrating adenosine receptors in the brain appeared from two other laboratories, using the nonselective agonist [^{3}H]CADO[27] or the N^6 derivative [^{3}H]R-PIA, later shown to be a somewhat A_1 selective agonist.[28] Like CHA, these two agonists had the advantage over adenosine of not being subject to degradation by the ubiquitous enzyme adenosine deaminase. Despite minor differences in results, all three laboratories made the surprising discovery that this brain receptor, widely distributed in the forebrain and other regions, appeared to be of the A_1 (R_i) type,[29, 30] a receptor subtype associated with a *decrease* in cyclic AMP, not the increase so readily studied in the previous functional assays. The cyclic AMP stimulatory adenosine effects were classified as the A_2 (R_a) type.
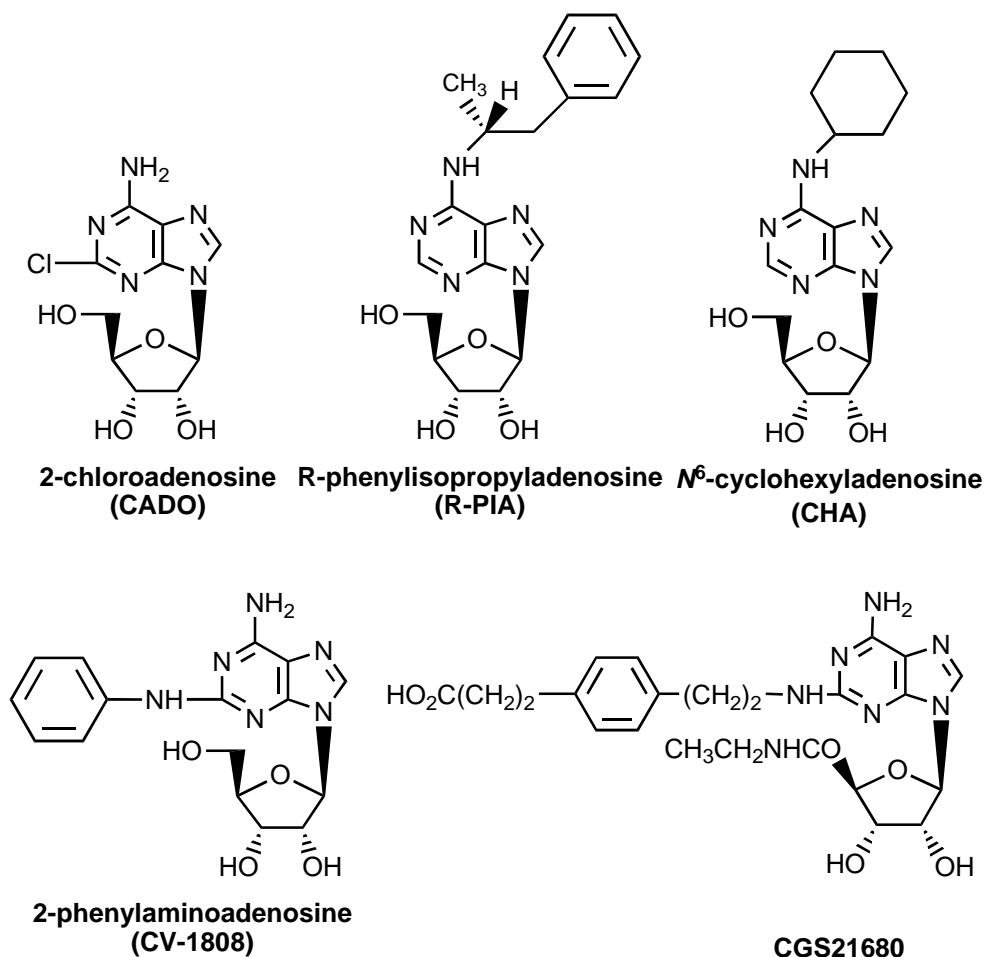
Figure 4. Structures of prototypical nucleoside derivatives studied as adenosine receptor agonists and as radioligands. CADO is nonselective, while certain other 2 substituted adenosine derivatives showed selectivity for the A_2 subtype. N^6 substituted adenosine derivatives generally showed selectivity for the A_1 subtype. [^{3}H]R-PIA and [^{3}H]CHA have been used as radioligands of nanomolar affinity for the A_1 subtype, and [^{3}H]CGS21680 is widely used as a high affinity radioligand selective for the A_{2A} subtype.

RECEPTOR SUBTYPES

These findings emphasized the presence in a single tissue of more than one adenosine receptor subtype. Even further complexity was apparent from a more careful analysis of the adenosine-mediated increase in cyclic AMP in the brain. The laboratories of Ted Rall and John Daly had noted that despite the readily demonstrable stimulation of cyclic AMP formation in brain slices, it was very difficult to show this response in a cell-free system [see Ref. 14]. Two reports that a stimulatory effect of adenosine analogues (and inhibition by methylxanthines) could be demonstrated in the basal ganglia[31] were soon confirmed in the Daly lab.[33] Here there was also a comparison to the response in slices, and it was noted that the adenosine analogues were much more potent in the homogenate preparation than in slices.[33] The relative potency of a number of agonists and antagonists did not differ; however, and it was only much later that two types of A_2 receptors (later called A_{2A} and A_{2B}) were clearly proposed.[34] The stimulatory response in

basal ganglia homogenate preparations was classified as an A_{2A} response (i.e., high affinity for typical agonists) and stimulatory response in brain slices corresponded to the A_{2B} (i.e., low affinity for typical agonists) subtype. The A_{2B} response was also detected in fibroblasts. This distinction provided a mechanistic framework that later led to new treatment concepts for diseases of the basal ganglia, such as Parkinsons disease.

MEDICINAL CHEMISTRY OF ADENOSINE RECEPTORS

The introduction of the first adenosine receptor binding assays to be used in conjunction with the functional assays of cyclic AMP that defined the various subtypes enabled the rapid growth of medicinal chemistry of these receptors beginning in the 1980s. John Daly was among the pioneers of this discovery effort through the synthesis of novel adenosine and heterocyclic derivatives, which were tested using the radioligand and assay techniques that he developed. For example, John Daly and coworkers introduced the first bioavailable A_2 selective antagonist DMPX (Figure 1).[39] Although the selectivity vs. A_1 receptors in rat was only moderate and depended on the assay method of comparison, it provided a critical pharmacological probe for distinguishing these subtypes.

CADO is a nonselective adenosine agonist (Figure 4), while certain other 2 substituted adenosine derivatives showed selectivity for the A_2 subtype. However, adenosine derivatives N^6 substituted with hydrophobic groups generally showed selectivity for the A_1 subtype. This distinction guided later SAR exploration by Daly and others. The 2 substituted adenosine derivative CV-1808 was the earliest example of an A_2 receptor selective agonist. Although the ratio in comparison to the A_1 subtype was small, it provided a lead for other 2-substituted adenosine analogues that are currently used as A_{2A} receptor selective agonists, such as the 5′-N-ethyluronamide derivative CGS21680.[46] Daly and coworkers demonstrated that CGS21680 is selective for the A_{2A} receptor and essentially inactive at the A_{2B} subtype.[40] John Daly and Ray A. Olsson at the University of South Florida systematically explored the SAR of the 2 position of adenosine to derive a family of A_{2A} receptor selective agonists,[41] as well as substitution at the N^6 position.[34] Daly and coworkers explored the truncation of the ribose moiety to methyl and other small alkyl groups,[42] which gave rise to a new class of A_1 adenosine receptor antagonists that was the precursor of a current clinical candidate. As the medicinal chemistry of adenosine receptors progressed and many other labs began to work in this area, John Daly explored chemically diverse heterocyclic structures as adenosine antagonists.[43] This exploration and categorization of potent nonxanthine antagonists gave rise to the precursor leads for many of the current selective and high affinity antagonists that are in preclinical studies. Thus, Daly and coworkers provided seminal studies of prototypical adenosine agonists and antagonists, which contributed to current therapeutic directions in the field.

Now four subtypes of adenosine receptors, A_1, A_{2A}, A_{2B} and A_3, have been cloned and pharmacologically characterized from several species including man,[35] and John Daly's vision that adenosine receptors may prove to be interesting drug targets[36] is receiving more and more attention both with respect to the CNS and in peripheral tissues.[37] John Daly's early review in the Journal of Medicinal Chemistry on adenosine receptor ligands as potential future drugs[36] became a citation classic, and his prediction is now coming of age. Currently, potent adenosine receptor agonists, antagonists, and other modulators are in clinical trials for inflammatory diseases, autoimmune diseases, cancer, neurodegenerative disorders, pain, arrhythmias, and in cardiac imaging.

John Daly's contributions to the adenosine receptor field were recognized with an award presented to him at the Conference on Purine Nucleosides and Nucleotides in Cell Signalling, held in Bethesda, MD on Sept. 17, 1989 for "pioneering research on the biology and chemistry of adenosine".[47]

ACKNOWLEDGMENT

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