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A_3-adenosine receptors: design of selective ligands and therapeutic prospects

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Introduction

Adenosine A_1 and A_2 receptors have been the subject of intense activity in the pharmaceutical industry. Adenosine agonists, which are almost exclusively derivatives of adenosine (1), have been sought as potential hypotensive (2), antipsychotic (3, 4), antiarrhythmic (5), antilipolytic (thus antidiabetic, 6) and cerebroprotective (7–10) agents. Adenosine antagonists, of which xanthines and a number of fused heterocyclic ring systems are representative (1), have been under development as antiasthmatic (11), antidepressant (12), antiarrhythmic (13), renal protective (14, 15), antiparkinson (16) and cognitive enhancing (17–19) drugs. In spite of the massive effort to develop selective ligands, a number of agents that initially looked promising did not survive clinical trials. One of the reasons for this failure has been the side effects, *e.g.*, an adenosine agonist Cl-936 (N^6-(2,2-diphenylethyl)adenosine), which showed efficacy in animal testing for antipsychotic-like activity (3), but caused arteriopathy in dogs, nausea, and other side effects (20).

Nevertheless, the interest in adenosine-based therapy has not waned. On the contrary, as our knowledge of the biological effects of this endogenous protective modulator (adenosine) advances, the envisioned therapeutic applications become more sophisticated and promising. Recently, the use of adenosine agonists in treating stroke has come into focus (9,10), since, for this acute application, the interference by some of the previously documented side effects would, in principle, be diminished. There may soon be clinical trials involving the use of A_1 agonists in treating stroke (21).

The discovery of a novel and distinct adenosine receptor subtype, the A_3 receptor, has opened new therapeutic vistas in the purine field. This receptor subtype has a unique pharmacological profile, distribution in the body, and effector coupling. Papers on selective A_3 agents are just beginning to appear (22), but clinical trials have not yet been attempted. It

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will take some time before the medicinal chemistry of A_3 receptors advances to the degree of selectivity already achieved for A_1 and A_{2a} receptors and before the physiological role of A_3 receptors is clarified.

Cloning of the A_3 receptor

Meyerhof *et al.* (23) cloned an orphan receptor from rat testes that resembled in sequence the known adenosine receptors. An identical clone from the rat brain was shown by the laboratories of Gary Stiles at Duke University and Olivier Civelli, then of University of Oregon, to function as an adenosine receptor (24) and was termed the A_3 receptor. This designation is not to be confused with an earlier tentative use of the same nomenclature to describe an unrelated phenomenon (25). This new receptor was unique in that adenosine had a very low affinity (initially estimated at 30 μM, see below) and its action was not antagonized by xanthines, such as theophylline, as are A_1 and A_2 receptors. Typical K_i values at A_3 receptors of roughly 10^{-4} M have been obtained (26, 27) for many xanthines that have nearly nanomolar potency at the other subtypes. A similar receptor, designated S17, was cloned from a sheep brain cDNA library by the laboratory of S. Reppert and identified by J. Linden and colleagues as an A_3-type receptor, *i.e.*, it inhibited adenylyl cyclase, and this action was xanthine-insensitive (28). However, the sheep A_3 receptor was only 72% homologous in sequence with the rat A_3 receptor. This low degree of homology (species homologues are usually in the 90–95% range) raises the question whether these clones represent a single subtype. The cloning from a human brain cDNA library of the sequence homologous to the sheep A_3 receptor was also reported (29).

Distribution of the A_3 receptor

The cloning of the A_3 receptor from species other than rat, *e.g.*, sheep (28) and human (29), has indicated that there are interspecies differences in its peripheral distribution. In the rat, the A_3 receptor has a very narrow distribution, being most highly expressed mainly in the testes, but also in lung, kidneys, heart, and brain. In the sheep, the A_3 receptor transcript is found in the lung, spleen, pars tuberalis, and pineal gland, with lower levels in the testes, kidneys, and brain (28). Curiously, the transcript was not detected in the sheep heart. The human A_3 receptor transcript (29) was most highly expressed in the lung and liver.

The distribution in the brain has been measured in absolute quantities through radioligand binding (2), and in relative levels in *in situ* hybridization experiments (24, 28, 30). There is a widespread, relatively low level of A_3 receptor binding sites throughout the mouse brain, with B_{max} values in the range of 120 fmol/mg protein (found in the striatum, cerebellum) to 220 fmol/mg protein (as seen in the hippocampus). In membranes from the forebrain, species differences in receptor density have also been found (31), with the B_{max} values of 29 (gerbil), 43 (rat), and 118 (rabbit) fmol/mg protein. In the rat brain (24), the transcript is weakly expressed in the cortex, striatum, and olfactory bulb. In the sheep brain (28), the transcript is modestly expressed in the cortex, striatum, hypothalamus, and cerebellum. Thus, preliminary indications are that irrespective of region, the densities are low, *i.e.*, comparable to the levels of nicotinic receptors in the brain (32), and roughly 10–30 fold lower than levels of cortical A_1 adenosine receptors or striatal A_{2a} receptors (22).

Second messenger systems

The A_3 receptor is coupled to at least two second messenger systems, inhibition of adenylyl cyclase (24) and stimulation of phospholipase C (33). When expressed in Chinese hamster ovary (CHO) cells, A_3 receptors from rat, sheep, or human are coupled to inhibition of adenylyl cyclase (24, 28, 29). Recently, it was shown using novel selective agonists that the A_3 receptor in rat brain slices activates phospholipase C in a GTP-dependent manner (34). The latter finding provides a possible mechanistic explanation for the effects of A_3 activation in stroke (see below), since this second messenger system has been implicated in the development of neuronal damage in stroke.

Ligand development

Selective A_3 agonists

Scores of adenosine derivatives have been synthesized as adenosine agonists (1). In general, modification of the N^6-position with hydrophobic moieties has provided selectivity for A_1 receptors, and substitution at the C2-position with amino-, oxo-ether, or alkynyl chains has resulted in A_{2a} selectivity. Replacement of the ring oxygen atom of the ribose moiety by a carbon or sulfur atom also provided a degree of A_{2a} selectivity (35).

We have found that many of the selective A_1 and A_{2a} agonists also have considerable affinity in binding at A_3 receptors (26). For example, the A_1-selective CPA (**12**, N^6-cyclopentyladenosine) is 2-fold more potent at A_3 *vs.* A_{2a} receptors (Table I). The A_{2a}-selective CGS21680 (**13**,2-[4-[(2 - carboxyethyl)phenyl]ethylamino] - 5′ - *N* - ethylcarboxyamidoadenosine) is 4.5-fold more potent at A_3 *vs.* A_1 receptors.

Is there now a general approach for designing adenosine derivatives with A_3 selectivity? We have reported that one principle of achieving A_3 selectivity in adenosine agonists is the combination of the optimal N^6- and 5′-substitutions (26). Specifically, among alkyl, cycloalkyl, and arylalkyl N^6-substituents, a benzyl group is favored, due to its diminished potency at A_1 and A_{2a} receptors. The A_3 selectivity-enhancing effects of N^6-benzyl modification are additive with the A_3 affinity-enhancing effects of the 5′-uronamido function, as in NECA (**9**, adenosine-5′-*N*-ethyluronamide). The first such hybrid molecule to show A_3 selectivity (26) was N^6-benzyl-NECA (**6**, Table I). In a comparison of various 5′-uronamido groups in mono-substituted adenosine derivatives, the 5′-*N*-methylamide (**8**, MECA) had particularly favorable A_3 receptor *vs.* A_1/A_{2a} affinity (36).

A study of substituent effects on the N^6-benzyl group has shown that substitution at the 3-position with sterically bulky groups, such as the iodo group, is optimal (36), leading to the development of the highly potent A_3 agonist N^6-(3-iodo-benzyl)-adenosine-5′-*N*-methyluronamide (**2**, IB-MECA, Table I) which is 50-fold selective for A_3 *vs.* either A_1 or A_2 receptors. A closely related, but less selective ligand containing radioactive iodine, [^{125}I]AB-MECA (**3**, N^6-(4-amino-3-iodobenzyl)-adenosine-5′-*N*-methyluronamide), was developed (37) for characterization of A_3 receptors and was found to have a K_d value of 3.6 nM in binding to rat A_3 receptors in the RBL-2H3 mast cell line. This radioligand has supplanted the use of the lower affinity [^{125}I]APNEA (radioiodinated N^6-[2-(*p*-aminophenyl)ethyl]adenosine) (24, 26) in our efforts to elucidate structure-activity

relationships (SAR). It is to be noted that APNEA (**10**), the noniodinated precursor which has been used in *in vivo* studies of A_3 receptors (see below), is actually 8-fold selective for A_1 receptors (38) (Table I). K_i values at the cloned rat A_3 receptors for the same compounds using as radioligand either [^{125}I]AB-MECA (**3**) or [^{125}I]APNEA, are quite comparable. [^{125}I]AB-MECA (**3**) is a suitable radioligand when used with transfected cell lines that only express the A_3 subtype. In studies of the brain, since this radioligand it is not highly selective for A_3 receptors it must be used in conjunction with an antagonist of A_1 and A_2 receptors. For this purpose, we have employed XAC, **20**, the xanthine amine congener (31), which in the rat effectively blocks only $A_1/A_{2a}/A_{2b}$ receptors at a concentration of 1 μM.

The influence of 2-substitution of adenosine on A_3 affinity was also studied (31). Initially it was found that 2-substituted analogs such as 2-chloroadenosine (**14**, CADO) and 2-chloro-N^6-cyclopentyladenosine (**11**, CCPA) were relatively well tolerated at A_3 receptors (26). For example, CPA (**12**) and CCPA (**11**) are equipotent at rat A_3 receptors (Table I). The C2-modification was also compatible or additive with A_3 potency-enhancing modifications at other sites on the adenosine molecule. In fact, upon combination with the previously elucidated N^6- and 5′-position modifications of IB-MECA (**2**), the 2-chloro group produced an even greater margin of A_3 selectivity. Thus, 2-chloro-IB-MECA (1, Cl-IB-MECA) displayed an A_3 selectivity in binding assays of 2500-fold *vs.* A_1 and 1600-fold *vs.* A_{2a} receptors. In the same study, the 2-methylthioether (4) and 2-methyl-amino (5) derivatives of IB-MECA were also shown to have both high selectivity and affinity for A_3 receptors. Thus, 2-substitution has indications of being a generally favored modification of A_3 selective agonists.

The affinity of adenosine at adenosine receptors is not readily measured directly in radioligand binding assays due to the need to add adenosine deaminase to destroy endogenous adenosine that lingers in most tissue preparations, regardless of extent of washing. In the study of Zhou *et al.* (24), the inhibition constant for adenosine was estimated as ~30 μM. However, we have estimated the affinity to be greater than originally proposed. Using a comparative method based on differences in affinity with various substitutions, we have estimated the K_i value to be close to 1 μM (Fig. 1). This has a highly significant bearing on the physiological role of A_3 receptors, since, under severe stress conditions, it is possible to exceed an endogenous concentration of adenosine of 1 μM, and thus (presumably) activate a large fraction of the A_3 receptors. A similar calculation for the same set of analogs binding to A_1 and A_{2a} receptors has estimated the K_i values of adenosine to be 10 and 30 nM, respectively.

The agonist structure-activity relationships, with emphasis on the N^6-benzyl ring substituents, were studied in a quantitative model using the Comparative Molecular Field Analysis (CoMFA) program in Sybyl (39). CoMFA studies suggested that the NH-CH_2 group at the N^6-position of IB-MECA could be replaced by O-NH or NH-NH without significant loss of A_3 affinity. Also in the same study, it was found that bulky chains located at the 3-position of the benzyl ring, which can adjust conformationally within the binding site of the receptor, were well tolerated in binding at A_3 receptors. The N^6-iodobenzyl group is so favorable towards binding at A_3 receptors that the mono-substituted N^6-

iodobenzyladenosine, **7**, is slightly selective for A_3 receptors (38). This is the only example reported so far of a mono-substituted adenosine analog with A_3 selectivity.

In order to define the structural requirements for molecular recognition by A_3 receptors, we have proposed a molecular model for ligand binding at this subtype (26) that is consistent with known structure-activity relationships. This model features anchoring of the ribose moiety of adenosine to a histidine residue in the seventh transmembrane helix, that is conserved among all adenosine receptor subtypes. Another histidine residue in the sixth transmembrane helix, also proposed to be involved in ligand recognition and common to A_1 and A_2 adenosine receptors, is absent in A_3 receptors. Thus, we verified our surmise that the affinity of xanthines at A_3 receptors could be enhanced by the presence of a ribose moiety.

In an effort to synthesize A_3 antagonists, we attempted to maximize the affinity of xanthine derivatives at the binding site. Molecular modeling (see below) followed by chemical synthesis suggested that one means of accomplishing this was to anchor the xanthines by adding a ribose group at the 7-position. Some members of this class of compounds, the 1,3-dialkyl-xanthine-7-ribosides, have been synthesized and were previously found by Uzerman and colleagues to bind to A_1 receptors (40). At rat brain A_3 receptors, 1,3-dibutylxanthine-7-riboside (**17**, DBXR) was found to bind with a K_i value of 6.03 μM (26), whereas the parent xanthine, 1,3-dibutylxanthine (**25**, Table II), displayed a K_i value of 143 μM. Thus, the presence of the ribose moiety enhances affinity of xanthines at rat A_3 receptors, presumably by interacting with the adenosine recognition region of the A_3 receptor, which is assumed to represent only a partial subset of the analogous region in A_1 receptors. At A_1 receptors there is a decrease in affinity in going from xanthines to xanthine-7-ribosides, since the xanthines themselves are of relatively high affinity, through recognition by a portion of the receptor that is missing from the A_3 receptor. As a hybrid between adenosine agonist and antagonist structures, DBXR (**17**) proved to be a partial agonist (26) in the A_3-mediated inhibition of adenylyl cyclase, as was reported previously for xanthine-7-ribosides acting at A_1 receptors.

Since 1,3-dibutylxanthine-7-riboside was nonselective, we explored the structure-activity relationships in this series of unnatural nucleosides in an effort to identify A_3 selective agents (41). Adding the same 5′-uronamide group (*N*-methylamide) that favored A_3 selectivity in adenosine derivatives had a similar effect in the xanthine-7-riboside series. 1,3 - Dibutylxanthine - 7 - riboside - 5′ - *N*- methylcarboxamide (**15**, DBXRM), with a K_i value of 229 nM at A_3 receptors, was 160-fold selective for rat A_3 *vs.* A_1 receptors and >400-fold selective *vs.* A_{2a} receptors (41). Although the intention was to identify A_3 antagonists, this derivative acted as a full agonist in the A_3 receptor-mediated inhibition of adenylyl cyclase, providing the first example of any non-adenosine derivative acting as a selective agonist at any subtype of adenosine receptors.

The parallel in SAR between adenosine derivatives and xanthine-7-ribosides is supportive of our A_3 receptor model which features the ribose moiety of the ligand, either adenosine or xanthine ribosides, coordinated by hydrogen bonding to the same amino acid residue, hypothetical, the histidine of the seventh transmembrane helix (26). There are also common features between the structure-activity relationships (27) for dialkylxanthines binding to A_3 receptors (see below) and xanthine-7-ribosides. The 1,3-dibutyl analogs (**17** and **25**, Table

II) in both cases contain the optimal chain length (for neutral molecules). A major difference is that for the xanthines, selectivity at the rat A_3 receptor was not achieved (27). At A_1 receptors, the xanthines are generally more potent than the corresponding xanthine-7-ribosides (26), while at rat A_3 receptors the converse is true.

Site-directed mutagenesis (42) has identified features of the A_1 receptor which when incorporated into A_3 receptor chimers provide high affinity binding of xanthines. Surprisingly, a region of the second extracellular loop (the C-terminal half) of the A_1 receptor had this property. Replacement of both this segment and the sixth and seventh transmembrane helices of the rat A_3 receptor with the bovine A_1 sequences resulted in a 50,000-fold increase in the affinity of CPX (**28**).

Species differences and A_3 antagonists

In the study of Linden *et al.* (28) it was noted that there were substantial species differences in the affinity of xanthines. As opposed to the rat A_3 receptor, which was described as xanthine-insensitive, the sheep and human receptors (28, 29) bound certain xanthines, especially those 8-aryl xanthines bearing a negative charge on the 8-position substituents such as BWA522 (**16**) and BW1433 (**19**), with considerably higher affinities (Fig. 2). Thus, the amine derivative XAC bound with K_i values of 181 and 71 nM at sheep and human A_3 receptors, respectively, and the carboxylic acid-containing xanthine BWA522 (**16**,3-(4-aminobenzyl) - 8 - [4 - [[[carboxy]methyl]oxy]phenyl] -1 - propyl-xanthine), bound with K_i values of 3 and 18 nM. At rat A_3 receptors, BWA522 was later found to bind with a K_i value of 1.17 mM (31). In general, the affinities of 8-arylxanthines at rat, rabbit, and gerbil brain A_3 receptors were considerably less (typically by nearly two orders of magnitude) than the previously reported affinities at cloned sheep and human A_3 receptors. This enhancement of affinity was present for a cationic (XAC, **20**) as well as anionic xanthines. 8-Cyclopentyl-1,3-dipropylxanthine (**28**, CPX) distinguished clearly the specificity of human (relatively high affinity, K_i 0.76 μM) *vs.* sheep (very low affinity, K_i 49 μM) A_3 receptors, with the affinity in the rat, rabbit and gerbil being intermediate.

The development of selective antagonists as pharmacological probes and radioligands for A_3 receptors remains a challenge. The cautious use of xanthines to define the physiological actions of A_3 receptor activation is essential. It is still undetermined whether these species differences justify proposing distinct (A_{3a} and A_{3b}) receptor subtypes (43).

The SAR of xanthines at rat A_3 receptors has been explored as well (27). The presence of a sulfonate (**21**), carboxylate (**16** and **18**) or multiple carboxylate (**23**) groups did not result in a significant enhancement of affinity at rat A_3 receptors (Table II), although as previously observed, an anionic group tended to diminish potency at A_1 and A_{2a} receptors. The rat A_3 receptor affinity was not highly dependent on the distance of a carboxylate group from the xanthine pharmacophore. 2-Thio (**18**) *vs.* 2-oxo (**22**) substitution favored A_3 potency, and 8-alkyl (**22**) *vs.* 8-aryl (**16**, **20**, and **21**) substitution favored A_3 selectivity, although few derivatives were truly selective for rat A_3 receptors. 1,3-Dimethyl-8-(3-carboxypropyl)-2-thioxanthine, **18**, was 7-fold selective for A_3 *vs.* A_{2a} receptors. 1,3,7-Trime-thyl-8-(*trans*-2-carboxyvinyl)xanthine, **24**, was somewhat selective for A_3 *vs.* A_1 receptors. For 8-aryl

xanthines, affinity at A_3 receptors was enhanced by 1,3-dialkyl substituents in the order dibutyl > dipropyl > diallyl. Curiously, xanthines having K_i values in the 1–20 μM range at rat A_3 receptors failed to antagonize the A_3 agonist-induced inhibition of adenylyl cyclase in transfected CHO cell membranes (27). At sheep A_3 receptors, however, at least one xanthine (100 μM BW1433, **19**) did antagonize the effects of NECA on adenylyl cyclase (22).

Interspecies differences in agonist affinity at A_3 receptors were less pronounced than those for xanthines (31). Among adenosine agonists of varied structure (5′-, 2-, and N^6-derivatives), the relative binding affinities at rat A_3 receptors are similar to those at human, but not sheep, A_3 receptors; however, in all species examined, the stereoselectivity for *R- vs.* S-PIA (N^6-(2-phenylisopropyl)adenosine), a well-characterized pattern at A_1 receptors, was preserved. The selectivity for the *R*-diastereomer was 6-fold at rat (26), 11-fold at sheep (28), and 10-fold at human A_3 receptors (29).

In vivo actions of A_3 receptor activation and therapeutic prospects

The first *in vivo* study of a selective A_3 agonist (22) indicated that it was a very potent locomotor depressant. The IC_{50} value for IB-MECA in open field behavior in mice was 16 μg/kg, i.p., but it was not as fully efficacious as either selective A_1 or A_{2a} agonists. Specifically, the A_1 and A_{2a} selective agonists caused nearly complete immobility (although not sedation), whereas the A_3 agonist could cause only a maximal 60% reduction in locomotor activity. The depression elicited by the A_3 agonist was not reversed, statistically significantly, by either an A_1 antagonist, CPX (**28**), or by a selective A_{2a} antagonist, 8-chlorostyrylcaffeine (**29**, CSC). In view of the subsequent studies of seizures and ischemia, the results were suggestive of a potent, central effect of A_3 activation.

Administration of IB-MECA in mice also caused rapid scratching behavior, of which the frequency of occurrence appeared to increase with the dose. Since activation of A_3 receptors facilitated release of histamine in a rat mast cell line (33), it was proposed that the scratching could be related to histamine. Coadministration of an H_1-histamine antagonist, cyproheptidine, eliminated this behavior. Thus, it appears that IB-MECA may release histamine *in vivo*. Compound 48/80, a mast cell releasing agent, administered *in vivo* mimics some of the effects of acutely administered A_3 agonists (44, 45).

Cardiovascular effects of A_3 receptor stimulation have been demonstrated indirectly (46), through coadministration of a nonselective agonist, APNEA (**10**, Table I), and an antagonist that blocks action only at A_1 and A_{2a} receptors. It appears that the hypotensive action of APNEA is related to a lowering of cardiac output, perhaps mediated by mast cell degranulation (45), and is not the result of vasodilatation, a well characterized effect of A_{2a} activation.

At our lab, we have studied the effects of IB-MECA (0.1 mg/kg, i.p.) on cardiovascular parameters (blood pressure, cerebral blood flow using a laser Doppler probe) in rats (Fig. 3) and gerbils (47). This dose corresponded to a relatively high dose in the locomotor study in mice (22), namely one that elicited a maximal depression in locomotor activity. The A_3 agonist administered alone caused a lowering of the blood pressure with little effect on heart rate. The pronounced hypotensive effect began within 2 min postinjection and was

maintained throughout the entire 90 min monitoring period. The effects of this agonist on blood flow and respiratory rates were not significant. In contrast, a potent and selective A_1 agonist, N^6-cyclopentyladenosine, **12**, at a dose of 0.1 mg/kg, i.p., caused an intense drop in both blood pressure (by 50%) and heart rate (by 20%) from their initial values. The A_1/A_2 antagonist BWA1433 (1,3-dipropyl-8-[4-(carboxyethynyl)phenyl]xanthine, 4 mg/kg i.p., **19**, Table II) did not reverse the hypotensive effects of IB-MECA. Blood flow and respiratory rate were increased by administration of the adenosine antagonist alone. IB-MECA alone or the coadministration of BWA1433 and IB-MECA had nearly no effect on body temperature.

A_3 agonists for cerebral ischemia

We have studied the effects of chronic *vs.* acute administration of selective adenosine agents, both agonists and antagonists, at the various subtypes. A_1 agonists given acutely are cerebroprotective in models of stroke and seizures. For A_1 receptors, there is a paradoxical reversal of the effects depending on either chronic or acute dosing regimen, as determined using spatial memory and seizure models. Thus, a chronically administered A_1 antagonist (CPX) was antiischemic and anticonvulsant, and a chronically administered A_1 agonist (CPA) was pro-cognitive (9).

A similar paradoxical reversal in cerebroprotection in gerbils was also seen for an A_3 agonist (Fig. 4). Chronically administered IB-MECA (100 μg/kg, i.p.) in gerbils dramatically improved the histopathological and neurological outcome after both 10 and 20 min ischemia induced by bilateral occlusion, and the survival rate was 90% compared to 60% in the controls. Moreover, the treatment preserved short-term memory following 10 min cerebral ischemia (47, 48). The acute administration of the same dose followed by ischemia resulted, on the other hand, in a more extensive deterioration of hippocampal cells, behavioral indicators, and decreased survival in treated *vs.* control animals.

Chronically administered IB-MECA was protective in chemically induced (NMDA or pentamethylenetetrazole) seizures (44). Significant improvement in seizure latency, neurological impairment, and survival was observed. In electrically induced seizures, chronic but not acute IB-MECA reduced postepileptic mortality.

It is unknown whether the protective effects of chronically administered IB-MECA or the opposite effects of acute IB-MECA are related to its effect on blood flow, neuronal mechanism, or both. Following chronically administered IB-MECA and stroke, postischemic cerebral reperfusion was significantly improved (47). Postischemic cerebral blood flow was greatly reduced following acutely administered IB-MECA (Fig. 5). Compound 48/80, which releases histamine and constricts arterioles, apparently diminishes the neurotoxic effects of peripherally administered NMDA or pentylenetetrazole, as does acutely administered IB-MECA (44). This suggests that the protective effects of acutely, but not chronically administered IB-MECA against chemically induced seizures may be related to vascular changes reducing the availability of the toxin in the brain.

A_3 agonists for cardiac preconditioning

Downey and coworkers have shown that the cardioprotective effect of a brief exposure to an adenosine agonist in rabbits prior to an ischemic infarct (preconditioning) is not reversed by some xanthines, *e.g.*, 200 nM CPX, that are known to block A_1 receptors, while another xanthine, SPT (**21**, 8-*p*-sulfophenyltheophylline), causes antagonism only at a high concentration of 100 μM (49). This has been interpreted as consistent with an A_3 component of preconditioning. Similar results were obtained in two other studies (50, 56). Thus, it is conceivable that exposure of the heart to a selective A_3 agonist might be highly protective.

The cell type on which A_3 receptors are located in the heart is not well established. The activation of A_3 receptors on mast cells has been proposed by Fozard and coworkers to lead to histamine release and subsequently hypertension (45). The presence on cardiomyocytes has not yet been demonstrated.

A_3 antagonists as antiinflammatory or antiasthmatic agents

Beaven *et al.* (52) have suggested that A_3 antagonists may have potential as antiinflammatory agents acting via mast cells. In human lung, A_3 receptors appear to be expressed mainly on eosinophils and possibly upregulated in pulmonary disease, suggesting a relevance to asthma (53). Adenosine was previously shown to be bronchoconstrictor in the asthmatic lung (54), although now, perhaps it appears to be acting through A_3 receptors. A selective A_3 antagonist might be therapeutically useful in treating asthma and other inflammatory disorders.

Other potential applications: reproduction, cancer, etc

Since A_3 receptors are expressed in testes (23) and appear to be involved in spermatogenesis, perhaps A_3 selective agents could be used in altering male fertility.

A number of other phenomena in which adenosine agonists produce a biological effect in a manner that is not antagonized by xanthines have been explained by invoking A_3 receptors. In these cases, the lack of a reliable antagonist, selective or otherwise, is a severe disadvantage. Adenosine was found to cause an increase in the serotonin uptake in RBL 2H3 cells (55), and this action was suggested to be of the A_3 type, although it was antagonized by XAC. Thus, there may be a connection between A_3 receptors and antidepressant drug therapy. There may be a connection with cancer, as well (56). It was proposed that the action of adenosine to inhibit the adhesion of killer lymphocytes to adenocarcinoma cells is through an A_3 receptor (56, 57). The implications of this finding for drug development are yet to be explored.

Summary

The A_3 receptor has been established as a distinct receptor subtype through cloning and the synthesis of selective agonists. The distribution of this subtype in the body is unique and more limited than for A_1 or A_2 receptors. Actions associated with this subtype include mast cell degranulation, cerebroprotection, cardioprotection, and possibly alteration of fertility. The effects on the inflammatory system and the high level of A_3 receptor expression in the

lungs suggests the use of A_3 antagonists for asthma. Thus, there is a tremendous potential for development for therapeutic purposes of selective drugs, either agonists or antagonists, acting at this receptor.

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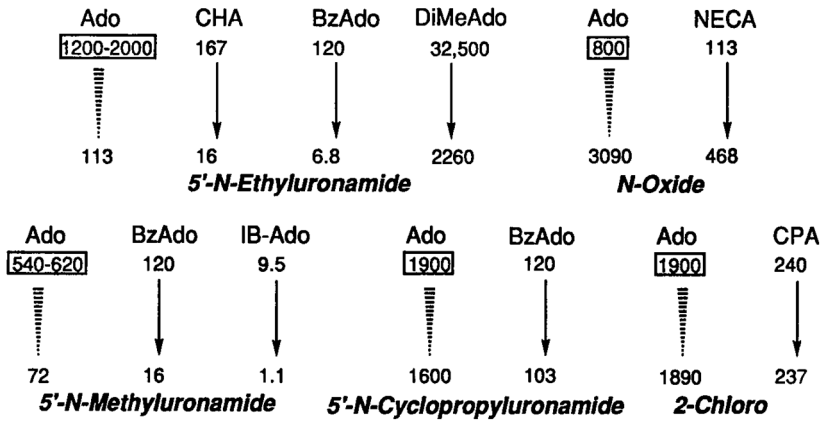


Fig. 1.

Estimation of the affinity of adenosine at rat A_3 receptors using an approach based on comparing K_i values for various adenosine analogs in radioligand binding assays. Estimation of affinity of adenosine at rat A_3 receptors was done by extrapolation from ratios of measured affinities of mono- and di-substituted analogs. The estimated value or range for the K_i of adenosine in nM is shown in a rectangular box for each group of compounds. K_i values (26,36, 38) in nM are shown for CHA (N^6-cyclohexyl), CPA (N^6-cyclopentyl), BzAdo (N^6-benzyl), DiMeAdo (N^6-dimethyl), IB-Ado (N^6-3-iodobenzyl), and NECA (5′-*N*-ethyluronamide) with or without the modification shown in italics. For example, the transformation of IB-Ado to the corresponding *N*-methyluronamide, *i.e.*, IB-MECA (second row of arrows, third entry, see Table I for structure), changes the K_i value from 9.5 to 1.1 nM. For the representative compounds selected, 5′-*N*-Et and 5′-*N*-Me uronamide modifications resulted in enhancement of affinity by 10- to 18-fold or 8-fold, respectively. 5′-*N*-Cyclopropyl uronamide and 2-chloro modifications did not markedly change affinities, and the N^1-oxide modification diminished affinity by 4-fold. Thus, the estimated affinity of adenosine is ~ 1 μM.

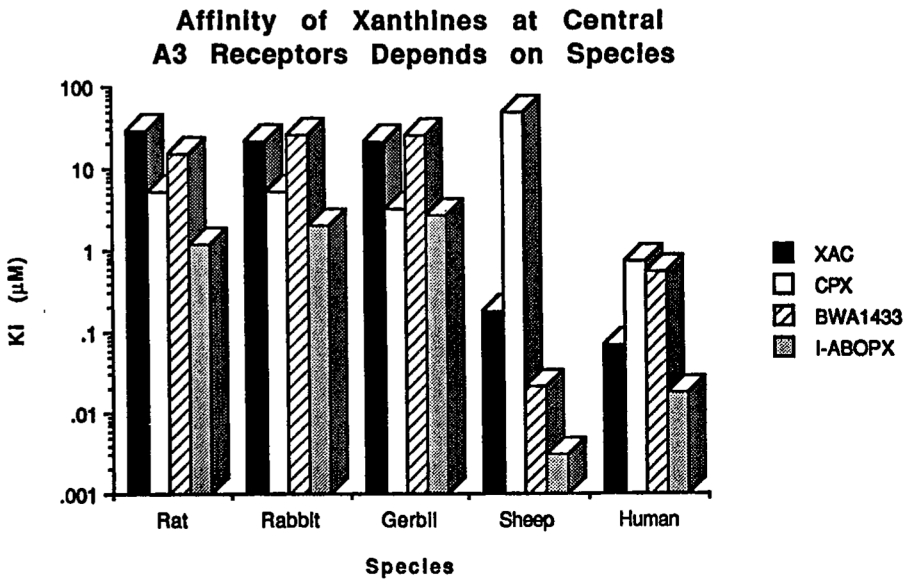


Fig. 2.

Species differences in the affinity of xanthine derivatives (for structures refer to Table II). The K_i values were determined in radioligand binding assays (*vs.* ^{125}I-labeled adenosine derivatives) in membranes of CHO expressing cloned A_3 receptors (28, 29, 31).

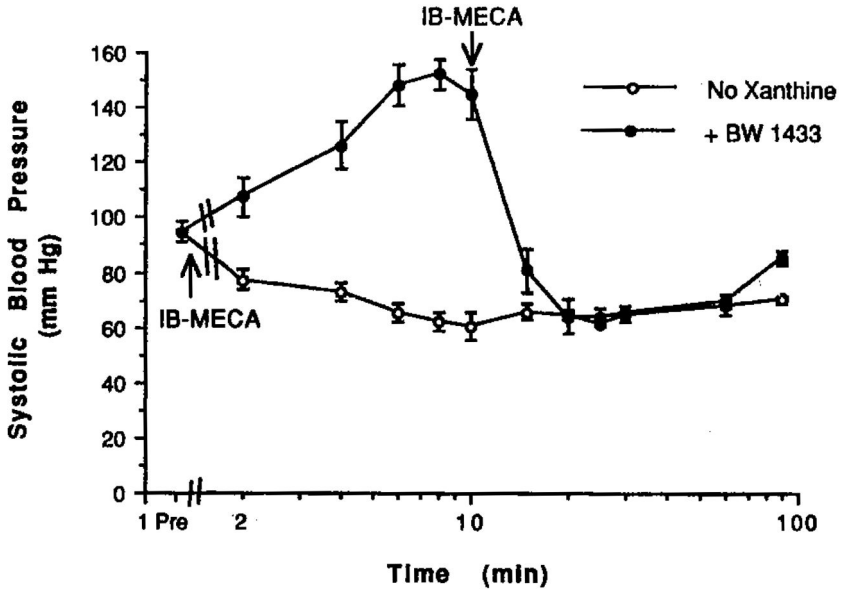


Fig. 3.

Effects on systolic blood pressure in rats. Rats (Sprague Dawley, 300–340 g) were lightly anesthetized using 1.5–2% halothane. They were monitored for heart rate with a tail sensor and blood pressure using the tail cuff method. Measurements were made at regular intervals: every 2 min for the first 10-min period, and every 5 min thereafter, until 30 min, at which time a 30-min interval was used, until 90 min. Each drug group had 5–7 animals. Drugs were dissolved in a vehicle consisting of a 80:20 mixture of Alkamuls EL-620 and saline, pH 7.4. For a 0.1 mg/kg dose, a solution of 2.5 mg/ml in vehicle was prepared. When administered alone, IB-MECA (0.1 mg/kg) was given at the beginning of the monitoring period. When coadministered with the antagonist BWA1433 (4 mg/kg), the antagonist was given at the beginning of the monitoring period, and IB-MECA (0.1 mg/kg) was given after 10 min.

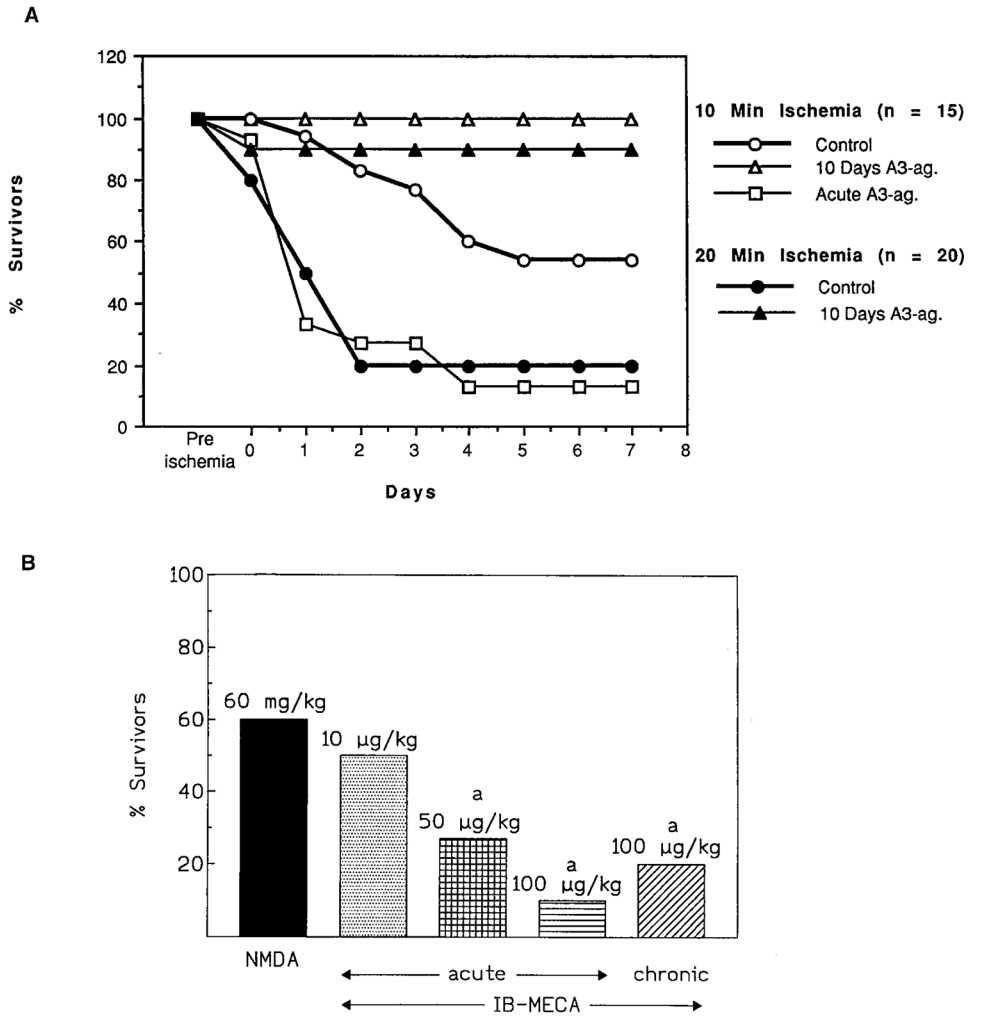


Fig. 4.

Effects of acute or chronic IB-MECA in gerbils. A) Survival in gerbils following 10 min bilateral carotid artery occlusion *i.e.*, global ischemia (47). IB-MECA (0.1 mg/kg) was injected i.p. either 15 min before ischemia (acute) or daily for 10 days prior to ischemia, with a 24-h gap between the last injected dose and the ischemia (chronic). B) Seizure mortality as a result of NMDA alone or NMDA following IB-MECA treatment (44). NMDA was administered at a dose of 60 mg/kg, i.p. IB-MECA at the dose indicated was injected i.p. either 15 min before NMDA (acute) or daily for 6 weeks prior to NMDA, with a 24-h gap between the last injected dose and the NMDA (chronic). Mortality was determined in the first 5 h following seizures. There was no change in mortality between 5 and 24 h.

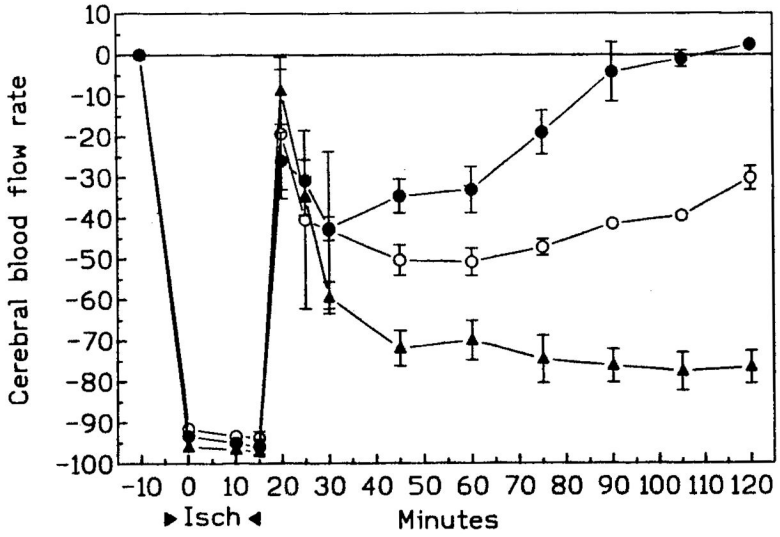
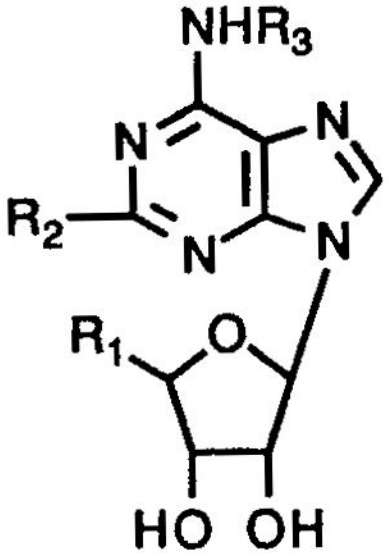


Fig. 5.

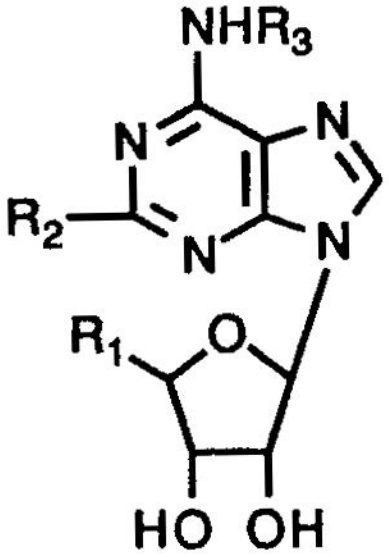
Cerebral blood flow rate (CBFR) in gerbils (n = 5/group) following either 10 min or 20 min bilateral carotid artery occlusion (47). Control gerbils are indicated by open circles. IB-MECA (0.1 mg/kg) was injected i.p. either 15 min before ischemia (acute, filled triangles) or daily for 10 days prior to ischemia, with a 24-h gap between the last injected dose and the ischemia (chronic, filled circles).

Table I

Affinities of adenosine derivatives at rat brain A_1, A_{2a}, and A_3 receptors, arranged in order of decreasing affinity at rat A_3 receptors.[a]



				K_i (μM)		
Compound	**R_1**	**R_2**	**R_3**	**$K_i(A_1)$**	**$K_i(A_{2a})$**	**$K_i(A_3)$**
1[b]	CH_3NHCO	Cl	3-I-Bz	0.82	0.47	0.00033
2[c]	CH_3NHCO	H	3-I-Bz	0.054	0.056	0.0011
3[d]	CH_3NHCO	H	3-I-4-NH_2-Bz	0.018	0.197	0.0013
4	CH_3NHCO	CH_3S	3-I-Bz	2.140	3.210	0.0023
5	CH_3NHCO	CH_3NH	3-I-Bz	4.89	4.12	0.00312
6[e]	EtNHCO	H	Bz	0.087	0.095	0.0068[l]
7	$HOCH_2$	H	3-I-Bz	0.02	0.0175	0.0095
8[f]	CH_3NHCO	H	H	0.0836	0.0668	0.072[l]
9[g]	EtNHCO	H	H	0.0063	0.0103	0.113[l]



Compound	R_1	R_2	R_3	K_i (μM)		
				$K_i(A_1)$	$K_i(A_{2a})$	$K_i(A_3)$
10[h]	$HOCH_2$	H	4-NH_2-$(CH_2)_2$	0.014	0.172	0.116
11[i]	$HOCH_2$	Cl	cyclopentyl	0.0006	0.95	0.237[l]
12[j]	$HOCH_2$	H	cyclopentyl	0.00059	0.462	0.24[l]
13[k]	EtNHCO	$NH(CH_2)_2$-φ-*p*-$(CH_2)_2$-COOH	H	2.6	0.015	0.584[l]
14	$HOCH_2$	Cl	H	0.0093	0.063	1.89[l]

[a] K_i ± SEM determined in radioligand binding assays expressed in μM (n = 3–6), using the following radioligands: A_1, [^{3}H]PIA in rat cortical membranes; A_{2a}, [^{3}H]CGS 21680 binding in rat striatal membranes; A_3, [^{125}I]AB-MECA binding, unless noted, in membranes of CHO cells stably transfected with the rat A_3-cDNA. A percent value indicates the percent displacement of radioligand at the concentration (M) given in parentheses.

[b] Cl-IB-MECA;

[c] IB-MECA;

[d] I-AB-MECA;

[e] Bz-NECA;

[f] MECA;

[g] NECA;

[h] APNEA;

[i] CCPA;

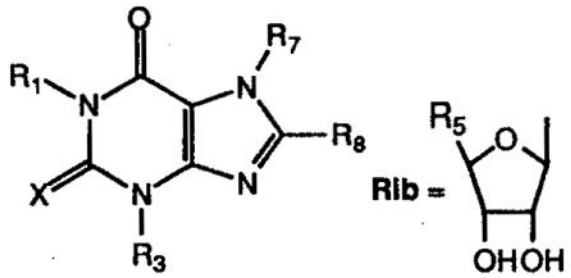
[j] CPA;

[k] CGS 21680.

[l] A_3 affinity determined *versus* [^{125}I]APNEA binding.

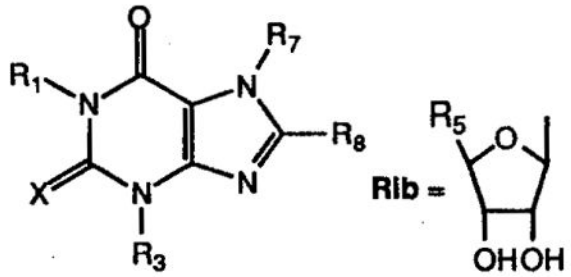
Table II

Affinities of xanthine derivatives at rat brain A_1, A_{2a}, and A_3 receptors, arranged in order of decreasing affinity at rat A_3 receptors.[a]



$R_1 = R_3$, X = O unless noted

Compound	R_1, R_3	R_8	R_7	K_i (μM) or % inhibition (conc.)		
				K_i (A_1)	K_i (A_{2a})	K_i (A_3)
15[b]	$CH_3(CH_2)_3$	H	Rib, R_5=MeNHCO	37.300	19% (10^{-4})	0.229
16[c]	1-$CH_3(CH_2)_2$ 3-CH_2(3-I-4-NH_2)	φ-*p*-OCH_2COOH	H	0.037	0.7	1.17
17[d]	$CH_3(CH_2)_3$	H	Rib, R_5-$HOCH_2$	4.190	19.500	6.03
18	CH_3 X = S	$(CH_2)_3COOH$	H	5.70	67.8	9.36
19[e]	$CH_3(CH_2)_2$	φ-*p*-CH=CHCOOH	H	0.015	0.80	15.0
20[f]	$CH_3(CH_2)_2$	φ-*p*-OCH_2CONH-$(CH_2)_2NH_2$	H	0.0112	0.063	29.0
21[g]	$CH_3(CH_2)_2$	φ-*p*-SO_3H	H	0.14	0.79	90.1
22	CH_3	$(CH_2)_3COOH$	H	0% (10^{-4})	25% (10^{-4})	93.4
23	$CH_3(CH_2)_2$	φ-*p*-OCH_2CONH-$(CH_2)_2NHCOCH_2N$-$(CH_2COOH)COCH_2CH_2$-COOH	H	2.86	9.22	99.9
24	CH_3	CH=CHCOOH	CH_3	3% (10^{-4})	42	130
25	$CH_3(CH_2)_3$	H	H	0.50	29.3	143[j]
26	CH_3	H	CH_3	29	48	30.1% (10^{-4})[j]
27	CH_3	H	H	8.5	25	23.1% (10^{-4})[j]
28[h]	$CH_3(CH_2)_2$	cyclopentyl	H	0.46	340	18.7% (10^{-5})[j]



$R_1 = R_3$, X = O unless noted

Compound	R_1, R_3	R_8	R_7	K_i (μM) or % inhibition (conc.)		
				K_i (A_1)	K_i (A_{2a})	K_i (A_3)
29[i]	CH_3	CH=CH-*m*-φCl	CH_3	28.2	0.054	4.2% (10^{-5})[j]

[a] K_i ± SEM determined in radioligand binding assays expressed in (μM (n = 3–6), using the following radioligands: A_1, [^{3}H]PIA in rat cortical membranes; A_{2a}, [^{3}H]CGS 21680 binding in rat striatal membranes; A_3, [^{125}I]AB-MECA binding, unless noted, in membranes of CHO cells stably transfected with the rat A_3-cDNA. A percent value indicates the percent displacement of radioligand at the concentration (M) given in parentheses.

[b] DBXRM;

[c] BWA522;

[d] DBXR;

[e] BWA1433;

[f] XAC;

[g] SPX;

[h] CPX;

[i] CSC;

[j] A_3 affinity determined *versus* [^{125}I]APNEA binding.