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N^6-substituted 9-methyladenines: a new class of adenosine receptor antagonists

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A series of 15 N^6-substituted 9-methyladenines have been assessed as antagonists of A_2-adenosine receptor-mediated stimulation of adenylate cyclase in membranes of human platelets and rat PC12 cells and of A_1-adenosine receptor-mediated inhibition of adenylate cyclases in membranes of rat fat cells and as inhibitors of binding of N^6-*R*-[^{3}H]phenylisopropyladenosine to A_1-adenosine receptors in rat brain membranes. N^6 substitution can markedly increase the potency of 9-methyladenine at A_1 receptors, while having lesser effects or even decreasing potency at A_2 receptors. Effects of N^6 substituents on adenosine receptor activity of the 9-methyladenines are reminiscent of effects of N^6 substituents on activity of adenosine, suggesting that N^6 substituted 9-methyladenines bind to adenosine receptors in the same orientation as do N^6-substituted adenosines. N^6-Cyclopentyl-9-methyladenine with K_i values at the A_1 receptors of 1.3 μM (fat cells) and 0.5 μM (brain) is at least 100-fold more potent than 9-methyladenine (K_i 100 μM, both receptors), while at the A_2 receptors K_B values of 5 μM (platelets) and 25 μM (PC12 cells) make it 5-fold more potent and equipotent, respectively, compared to 9-methyladenine (K_B 24 μM, both receptors). N^6-Cyclopentyl and several other N^6-alkyl and N^6-cycloalkyl analogs are selective for A_1 receptors while 9-methyladenine is the most A_2 receptor selective antagonist. The N^6-*R*- and N^6-*S*-(1-phenyl-2-propyl)-9-methyladenines, analogous to N^6-*R*- and N^6-*S*-phenylisopropyladenosines, exhibit stereoselectivity at both A_1 and A_2 receptors. Marked differences in potency of certain N^6-substituted 9-methyladenines at the A_2 receptors of human platelets and rat PC12 cells provide evidence that these are not identical receptors.

Adenosine receptor; Adenylate cyclase

1. INTRODUCTION

Adenosine receptors have been divided into two subtypes, based on adenylate cyclase activity: A_1 (R_i) receptors mediate inhibition and A_2 (R_a) receptors mediate stimulation of adenylate cyclase activity (reviews [1,2]). Some N^6-substituted adenosine analogs like N^6-*R*-1-phenyl-2-propyladenosine (*R*-PIA) have very high affinity for A_1-adenosine receptors, while 5'-*N*-ethylcarboxamidoadenosine (NECA) is more potent than N^6-substituted analogs at A_2 receptors. Alkylxanthines, such as caffeine and theophylline, are the best known antagonists at adenosine receptors. Adenine was generally believed to have no effect on adenosine receptor-controlled systems. However, adenine is a specific, competitive antagonist of adenosine-induced cyclic AMP accumulation in a human fibroblast cell line with a K_i of 200 μM [3]. Methylation of adenine at the 9-position increases potency about 4-fold. A variety of N^6-substituted 9-methyladenine derivatives

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have now been prepared and tested in three adenylate cyclase-coupled adenosine receptor systems. For A_2-adenosine receptors human platelets and rat pheochromocytoma (PC12) cells and for A_1 receptors rat fat cells were used. In addition, the affinity for the A_1-binding site for N^6-*R*-1-[^{3}H]phenylpropyladenosine ([^{3}H]PIA) was determined in rat brain membranes. Certain of the N^6-substituted 9-methyladenines proved to be potent antagonists at adenosine receptors and some showed selectivity for either A_1 or A_2 receptors.

2. MATERIALS AND METHODS

The synthesis and the chemical properties of the adenine and hypoxanthine derivatives will be described elsewhere. [α-^{32}P]ATP (40 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). N^6-*R*-1-[^{3}H]Phenyl-2-propyladenosine ([^{3}H]PIA, 49.9 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Other compounds used in this study were from standard sources as described [4].

Human platelet, rat pheochromocytoma (PC12) cell, rat fat cell and rat cerebral cortex membranes were prepared as in [4–6]. Adenylate cyclase activity and binding of [^{3}H]PIA to cerebral cortex membranes were determined essentially as described [4–6].

K_B values for the compounds were determined as in [4]. Briefly stated, concentration-response curves of NECA for the stimulation of adenylate cyclase of PC12 cell and platelet membranes and of *R*-PIA for the inhibition of isoproterenol-stimulated adenylate cyclase activity in fat cell membranes in the absence and presence of the adenine derivative were done using at least 7 concentrations of the agonist. EC_{50} and IC_{50} values for the agonists were obtained from the concentration-response curves by linear regression after logit-log transformation. K_B values of the antagonists were calculated using the Schild equation $K_B = C/(CR - 1)$, where C denotes the concentration of the competitor and CR the ratio of the EC_{50} and IC_{50} values in the presence and absence, respectively, of the competitor. IC_{50} values of the compounds for inhibition of [^{3}H]PIA binding to cerebral cortex membranes were transformed into K_i values as described [6].

3. RESULTS

3.1. *A_2-Adenosine receptors*

The effects of adenine and adenine analogs on A_2 receptor were studied in human platelets. In these cells, A_2 receptor-mediated stimulation of adenylate cyclase results in an inhibition of aggregation [5,7].

Adenine (compound **1**) itself does not affect basal adenylate cyclase activity (not shown), but antagonizes the NECA-induced stimulation of adenylate cyclase activity (table 1). However, adenine (**1**) is a very weak antagonist at A_2 receptors of platelets, with a K_B value of 760 μM (table 1). Incorporation of a methyl group at the 9-position of the adenine molecule results in a marked increase in potency. Thus, 9-methyladenine (**2**) is 30-fold more potent than the adenine itself at A_2 receptors of platelets.

Substituents at the N^6-position of 9-methyladenine (**2**) markedly influence the antagonist potency at the platelet A_2 receptor. The N^6-cycloalkyl analogs (**3,4,6**) are more potent than 9-methyladenine itself.

Incorporation of an additional methyl group into N^6-cyclopentyl-9-methyladenine (**4**) so as to yield a tertiary carbon at the N^6-nitrogen reduces potency with N^6-(1-methylcyclopentyl)-9-methyladenine (**5**) being about 10-fold less potent than the parent cyclopentyl analog (**4**). The N^6-methyl analog (**7**) is much less potent than 9-methyladenine at the platelet receptor, while the N^6-3-pentyl analog (**8**) is 2.5-fold more potent. The N^6-phenyl analog (**9**) is equipotent to 9-methyladenine. The presence of the *ortho*-fluoro moiety in compound **10** increases potency 2-fold at the platelet A_2 receptor. The N^6-benzyl and N^6-2-phenethyl analogs (**11,12**) are less potent than 9-methyladenine at the platelet receptors. N^6-2-(3,4,5-Trimethoxyphenylethyl)-9-methyladenine (**13**) is as potent as 9-methyladenine. The heteroaryl analog N^6-2-(3-pyridylethyl)-9-methyladenine (**14**) is 4-fold less potent than 9-methyladenine, while another heteroaryl analog N^6-2-(3-thienylethyl)-9-methyladenine (**15**) is somewhat more potent. The N^6-1-phenyl-2-propyl derivatives are analogs containing a chiral carbon attached to the N^6-nitrogen: The *R*-isomer (**16**) is only about 1.7-fold more potent than the *S*-isomer (**17**). The O^6-phenyl derivatives of 9-methylhypoxanthine

Table 1
Potencies of 9-methyladenines and related compounds as antagonists at A_2- and A_1-adenosine receptors

Compound	Adenylate cyclase			Binding
	K_B (μM) vs NECA stimulation		K_B (μM) vs PIA inhibition	K_i (μM) vs [^{3}H]PIA
	(Human platelet membranes)	(Rat PC12 membranes)	(Rat fat cell membranes)	(Rat brain membranes)
1. Adenine	760 (610–950)	570 (330–990)	>1000	>100 (28%)
2. 9-Methyladenine (9-MA)	24 (21–27)	24 (19–30)	112 (79–160)	106 (87–129)
N^6-substituted 9-methyladenines				
3. N^6-Cyclobutyl-9-MA	5.5 (2.4–13)	23 (14–39)	0.89 (0.76–1.1)	1.2 (1.1–1.5)
4. N^6-Cyclopentyl-9-MA	4.9 (4.5–5.4)	25 (17–36)	1.3 (0.88–2.0)	0.54 (0.45–0.67)
5. N^6-Methylcyclopentyl-9-MA	45 (37–53)	56 (37–85)	9.0 (5.6–14.2)	2.5 (1.9–3.2)
6. N^6-Cyclohexyl-9-MA	7.4 (2.2–25)	21 (12–38)	0.65 (0.44–0.96)	0.94 (0.40–2.2)
7. N^6-Methyl-9-MA	150 (120–200)	130 (80–210)	220 (150–310)	>100 (42%)
8. N^6-3-Pentyl-9-MA	11 (9.4–12)	53 (35–78)	7.6 (6.4–9.1)	3.3 (2.2–5.1)
9. N^6-Phenyl-9-MA	21 (4.5–98)	107 (60–190)	10 (5.5–18)	25 (13–49)
10. N^6-2-Fluorophenyl-9-MA	11 (6.6–20)	29 (24–35)	17 (11–27)	8.5 (7.1–10.2)
11. N^6-Benzyl-9-MA	57 (37–89)	100 (77–130)	49 (32–75)	17 (7.5–36)
12. N^6-2-Phenethyl-9-MA	170 (140–220)	120 (90–160)	>300	>100 (12%)
13. N^6-2-(3,4,5-Trimethoxyphenylethyl)-9-MA	23 (21–25)	40 (38–42)	122 (93–159)	>100 (43%)
14. N^6-2-(3-Pyridylethyl)-9-MA	92 (80–107)	117 (83–147)	96 (70–130)	41 (32–54)
15. N^6-2-(3-Thienylethyl)-9-MA	14 (4.4–45)	25 (4.9–102)	24 (20–30)	20 (14–27)
16. N^6-*R*-1-Phenyl-2-propyl-9-MA	13 (8–22)	25 (19–33)	7.2 (5.5–9.5)	2.5 (1.9–3.4)
17. N^6-*S*-1-Phenyl-2-propyl-9-MA	23 (18–28)	74 (43–128)	23 (11.4–47)	10 (6–17)
O^6-substituted 9-methylhypoxanthines				
18. O^6-Phenyl-9-methylhypoxanthine (9-mH)	>1000	>1000	400 (370–470)	>100 (10%)
19. O^6-(2-Fluorophenyl)-9-MH	>1000	>1000	400 (310–510)	>100 (11%)
20. O^6-(3-Fluorophenyl)-9-MH	370 (150–190)	>1000	>1000	>100 (16%)
21. O^6-(4-Fluorophenyl)-9-MH	>1000	>1000	560 (470–680)	>100 (20%)

K_B and K_i values were calculated as described in section 2 and are geometric means with 95% confidence limits from three experiments. In some cases, the inhibition of binding at the highest concentration tested is given in parentheses following that concentration

(18–21) are very weak or inactive as inhibitors of NECA-induced stimulation of adenylate cyclase activity of human platelet membranes.

The potencies of the adenine derivatives were determined in a similar manner for A_2 receptors of rat pheochromocytoma (PC12) cells [4,8,9], using antagonism of the NECA-induced stimulation of adenylate cyclase activity of the PC12 cell membranes to assess antagonist potencies (table 1). While there are similarities, there are also some notable differences in the structure-activity relationship for the adenines at A_2 receptors of platelets and PC12 cells.

As was the case for the platelet system, adenine **(1)** is a very weak antagonist of the NECA-stimulated adenylate cyclase in PC12 cell membranes with a K_B of 570 μM. 9-Methyladenine **(2)** is equally potent at A_2 receptors of human platelets and rat PC12 cells with a K_B of about 25 μM in both cases. In contrast to the results with platelets, incorporation of N^6 substituents into 9-methyladenine does not in any case increase the potency

of the 9-methyladenine at the A_2 receptors of PC12 cells:

Potencies of all of the N^6-substituted 9-methyladenines at A_2 receptors of PC12 cells are either the same as or lower than that of the parent compound.

In certain cases, namely the N^6-cyclopentyl (**4**), N^6-3-pentyl (**8**) and N^6-phenyl (**9**) analogs, the analog is 5-fold more potent at the platelet A_2 receptor than at the PC12 A_2 receptor. In no case is the N^6-substituted 9-methyladenine less potent at the platelet A_2 receptor than at the PC12 A_2 receptor. Incorporation of an additional methyl to yield the tertiary analog N^6-1-methylcyclopentyl-9-methyladenine (**5**) reduces potency only 2-fold in PC12 cells, while decreasing potency nearly 10-fold in platelets.

The *R*- and *S*-isomers of N^6-1-phenyl-2-propyl-9-methyladenine (**16,17**) exhibit an *R/S* ratio of 3 in PC12 cells compared to a ratio of 1.7 in platelets. The O^6-phenyl-9-methylhypoxanthines (**18–21**) are nearly inactive in both cell types.

3.2. *A_1-Adenosine receptors*

Rat fat cells were used for evaluation of structure-activity relationships of adenine derivatives at adenylate cyclase-coupled A_1-adenosine receptors. In these cells, adenosine analogs cause an inhibition of adenylate cyclase activity and lipolysis [10].

Adenine (**1**) itself does not affect *R*-PIA-induced inhibition of fat cell adenylate cyclase activity (table 1). 9-Methyladenine (**2**) antagonizes the effect of *R*-PIA with a K_B of 112 μM and is, therefore, about 5-fold less potent at A_1 receptors than at A_2 receptors. Incorporation of cycloalkyl or alkyl substituents into the N^6-position of 9-methyladenine (**2**) can markedly increase the antagonistic potency at the fat cell A_1 receptor. Thus, the N^6-cycloalkyl-9-methyladenines (**3,4,6**) are about 100-fold more potent than the parent compound 9-methyladenine and N^6-3-pentyl-9-methyladenine (**8**) is about 15-fold more potent than the parent compound at A_1 receptors of fat cells. N^6-Methylcyclopentyl-9-methyladenine (**5**) is about 7-fold less potent than the N^6-cyclopentyl analog (**4**). The N^6-methyl analog (**7**) is 2-fold less potent than 9-methyladenine. The two N^6-phenyl analogs (**9,10**) are 6–10-fold more potent than 9-methyladenine in the fat cell, while the N^6-benzyl (**11**) analog is only 2-fold more potent. The N^6-2-phenethyl (**12**) analog is much less potent. Of the phenethyl (**12,13**) and heteroarylethyl (**14,15**) analogs only the N^6-2-(3-thienylethyl)-9-methyladenine is more potent than 9-methyladenine itself in the fat cell. The *R*- and *S*-isomers of N^6-1-phenyl-2-propyl-9-methyladenine (**16,17**) exhibit a 3-fold stereoselectivity in fat cells, which is the same as in the PC12 cells. However, in contrast to PC12 cells, these *R*- and *S*-isomers (**16,17**) are about 16- and 5-fold, respectively, more potent than the parent compound at A_1 receptors of fat cells. The O^6-phenyl-9-methylhypoxanthines (**18–21**) are very weak or inactive as antagonists in fat cell membranes.

Similar results were obtained when the K_i values of the adenine derivatives for inhibition of [^{3}H]PIA binding to rat cerebral cortex membranes were determined (table 1). The low potency of adenine (**1**) is commensurate with the results from the fat cell adenylate cyclase assay. 9-Methyladenine (**2**) is equally potent at A_1 receptors of fat cells and cerebral cortex. The K_i values of the N^6-substituted 9-methyladenine derivatives for inhibition of radioligand binding in brain membranes are generally 2- and 3-fold lower than the corresponding K_B values from the fat cell adenylate cyclase. As in the case with fat cells, N^6-cyclopentyl-9-methyladenine (**4**) is about 5-fold more potent than the N^6-methylcyclopentyl analog (**5**). The 2-phenethyl (**12,13**) analogs are very weak antagonists of [^{3}H]PIA binding in rat brain membranes as expected from results with fat cell adenylate cyclase. N^6-*R*-1-Phenyl-2-propyl-9-methyladenine (**16**) has a K_i of 2.5 μM vs [^{3}H]PIA binding and is, therefore, 4-fold more potent than the *S*-isomer (**17**). The O^6-phenyl-9-methylhypoxanthine derivatives (**18–21**) only marginally inhibit [^{3}H]PIA binding.

4. DISCUSSION

Xanthines, the major structural class of antagonists for adenosine receptors, have a planar heterocyclic ring system analogous to the heterocyclic purine (adenine) ring of adenosine. It has been proposed that the site in adenosine receptors that interacts with the adenine ring of adenosine also interacts with the xanthine ring of such adenosine antagonists as theophylline and

caffeine [2]. A variety of other compounds containing a planar heterocyclic ring have antagonistic activity at adenosine receptors. These include pyrazolopyrimidines [11], pyrazolopyridines [12, 13], mesoionic xanthine analogs [14], benzopteridines [3], and 9-methyladenine [3]. The last heterocycle 9-methyladenine, because of the identity of the heterocyclic ring with that of adenosine, seems even more likely than other heterocycles to bind at the same 'heterocycle' site as do the adenosines. The present study was designed to test the premise that, as in the case of adenosine, N^6 substituents on 9-methyladenine would alter activity of the 9-methyladenines in the same way as N^6-substituents alter the activity of adenosines. The topography of binding site for N^6 substituents in both A_1- and A_2-adenosine receptors has been extensively investigated (see [9,16] and references therein). The binding site for N^6 substituents differs significantly for A_1 receptors compared to A_2 receptors. At the A_1 receptors, N^6 substituents can markedly enhance activity. The stereoselectivity for compounds such as *R*- and *S*-PIA that contain chiral N^6 substituents is a well-known characteristic of A_1 receptors. At the A_2 receptors, most N^6 substituents reduce activity of adenosine and stereoselectivity is less pronounced than at A_1 receptors.

Certain N^6 substituents do markedly enhance activity of 9-methyladenine at A_1 receptors. The N^6-cycloalkyl-9-methyladenines (**3,4,6**) are the most potent of N^6-substituted 9-methyladenines at A_1 receptors being 80–200-fold more potent than 9-methyladenine. Similarly, N^6-cycloalkyladenosines are among the most potent N^6-substituted adenosines of A_1 receptors [9]. Introduction of an additional methyl to N^6-cyclopentylmethyladenine (**4**) to yield N^6-1-methylcyclopentyl-9-methyladenine (**5**) reduces activity at A_1 receptors. Similarly, activity of the N^6-(1-methylcyclopentyl)adenosine at A_1 receptors is reduced compared to N^6-cyclopentyladenosine [9]. The only N^6-alkyl or N^6-cycloalkyl substituent that reduces activity of 9-methyladenines at A_1 receptors is methyl, reminiscent of the low activity of N^6-methyladenosine at A_1 receptors [9]. The modest activity of N^6-benzyl-9-methyladenine (**11**) is also consonant with the low activity of N^6-benzyladenosine at A_1 receptors [9]. Somewhat surprising was the low activity of N^6-2-phenethyl-9-methyladenine (**12**), since the corresponding adenosine analog exhibits moderate activity at A_1 receptors [9]. The *R*- and *S*-enantiomers of N^6-(1-phenyl-2-propyl)-9-methyladenine, analogous to *R*- and *S*-PIA, exhibit stereoselectivity at A_1 receptors with the *R*-enantiomer being 3–4-fold more potent than the *S*-enantiomer. Unlike the diasteromers *R*- and *S*-PIA, these analogs are true enantiomers, since the other chiral centers of the ribose moiety are absent. It should be noted that the stereoselectivity of the 9-methyladenine analogs at A_1 receptors (table 1) is much less than that of *R*- and *S*-PIA at A_1 receptors [9].

The results indicate that 9-methyladenines show effects of N^6 substituents on activity at A_1 receptors similar to, but not identical with the effects of N^6 substituents on agonist activity of adenosines at A_1 receptors. The near lack of activity of the four O^6-phenyl-substituted 9-methylhypoxanthines is reminiscent of the inactivity of purine ribosides containing oxygen or sulfur in place of nitrogen at the 6-position at adenosine receptors [15].

At A_2 receptors, N^6 substituents have much smaller effects on activity of 9-methyladenine than was the case of A_1 receptors. Indeed, many substituents have no effect or reduce activity. The two A_2 receptors do not appear identical in terms of interaction with the N^6-substituted 9-methyladenines. Whether such differences are related to species or tissue are unknown. Certainly, brain A_1 receptors differ markedly in agonist/antagonist activity in different species [16]. At the A_2 receptor of human platelets only the cycloalkyl-, 3-pentyl-, 2-fluorophenyl-, 2-(3-thienylethyl)- and *R*-1-phenyl-2-propyl- substituents enhance activity. Certain N^6-substituted adenosines corresponding in structure to the N^6-substituted 9-methyladenines have been investigated as agonists at platelet A_2 receptors [9]. There was only a modest range of potency with the N^6-cyclobutyl-, N^6-cyclohexyl-, N^6-2-(3-thienylethyl)- and N^6-benzyladenosines and *R*-PIA being the more potent of the N^6-substituted adenosines. Thus, the results with N^6-substituted 9-methyladenines at platelet A_2 receptors would not have been predicted from the effects in the analogous adenosines. At the A_2 receptors of PC12 cells, none of the N^6 substituents increased activity relative to 9-methyladenine itself. Indeed, certain substituents decreased activity. Again, these effects would not have been

predicted from the agonist activity of the analogous N^6-substituted adenosines at A_2 receptors of PC12 cells [9]: As for the platelet A_2 receptor there was not a wide range of potencies for the adenosines at PC12 receptors with the N^6-cyclobutyl-, *R*-PIA, N^6-2-phenethyl-, N^6-cyclohexyl-, N^6-2-(3-pyridylethyl)- and N^6-2-(3,4,5-trimethoxyphenylethyl) analogs being the more potent of the series. Thus, the effects of N^6 substitution on activity of 9-methyladenines and adenosines at A_2 receptors are not identical, perhaps reflecting the lack of major positive contributions of such substituents to activity at A_2 receptors. It is of interest that data on both the antagonist series of N^6-substituted adenine (table 1) and the agonist series of N^6-substituted adenosines [9] provide evidence for the lack of identity of A_2 receptors in platelets and PC12 cells. The O^6-phenyl-9-methylhypoxanthines are inactive or nearly so at the A_2 receptors, as is the case for 6-phenoxypurine riboside at coronary A_2 receptors [17].

Certain of the N^6-substituted 9-methyladenines are somewhat selective (5–10-fold) for A_1 receptors, in particular N^6-cyclobutyl, cyclopentyl-1-methylcyclopentyl- and cyclohexyl analogs, while 9-methyladenine and N^6-2-(3,4,5-trimethoxyphenylethyl)-9-methyladenine exhibit a 3–4-fold selectivity for A_2 receptors. Further investigation of this new class of adenosine receptor antagonists both in vitro and in vivo will be required to establish their usefulness in definition and elucidation of functions of adenosine receptors.

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