Notes

Novel N^6-(Substituted-phenylcarbamoyl)adenosine-5′-uronamides as Potent Agonists for A_3 Adenosine Receptors

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A series of adenosine-5′-uronamide derivatives bearing N^6-phenylurea groups have been synthesized and tested for their affinity at A_1 and A_{2A} adenosine receptors in rat brain membranes and at cloned rat A_3 receptors from stably transfected CHO cells. Some N^6-arylcarbamoyl derivatives, N^6-((2-chlorophenyl)carbamoyl)-, N^6-((3-chlorophenyl)carbamoyl)-, and N^6-((4-methoxyphenyl)carbamoyl)adenosine-5′-ethyluronamide (**4l**−**n**), were found to have affinity at A_3 receptors in the low nanomolar range (K_i values < 10 nM). In CHO cells stably transfected with the rat A_3 receptor, compound **4n** was found to be a full agonist in inhibiting adenylate cyclase activity. The present study represents the first example of N^6-acyl-substituted adenosine analogs having high affinity at adenosine receptors and, in particular, at the A_3 receptor subtype.

Introduction

Adenosine receptors mediate a wide variety of actions of the local modulator adenosine in the nervous, cardiovascular, renal, immune, and other systems.[1]

Four subtypes of adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) have been defined on the basis of cloned sequences[2−5] and on pharmacological distinctions. The A_1 and A_2 receptor subtypes are linked to inhibition and stimulation, respectively, of adenylyl cyclase.[6−8] A_3 receptors are linked to both the stimulation of phospholipase C in RBL-2H3 mast cells[9] and in rat brain slices[10] and the inhibition of adenylyl cyclase.[5]

Many selective agonists and antagonists have been developed for the A_1[11−16] and A_{2A}[17−20] receptor subtypes. Some of these have shown promise as potential therapeutic agents in the treatment of hypertension,[18] Parkinson's disease,[21] cognitive deficits,[22] schizophrenia,[23] epilepsy, and renal failure.[24] Selective and/or high-affinity agonists and antagonists for the A_{2B} receptor have not yet been reported. The A_3 receptor has recently been cloned from a rat brain cDNA library.[5] Although selective antagonists are not yet available,[25] highly selective agonists have been reported.[26−30] Among these agonists is IB-MECA (N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide) which has a K_i value of 1.1 ± 0.3 nM at rat A_3 receptor and 50-fold selectivity vs either A_1 or A_{2A} receptors.[28,29] The radioligand [^{125}I]AB-MECA (N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide)[31] has become useful in screening new derivatives at cloned rat A_3 receptors expressed in CHO cells. There is a large difference in sequence and pharmacological properties between A_3 receptors found in rat and those of human[32] and sheep.[33] Chronically administered IB-MECA has been found to protect against cerebral ischemia in gerbils[34] and chemically induced seizures in mice.[35] In addition to the protective effects in the central nervous system, A_3-selective agents have been postulated to be useful in cardioprotection (agonists)[36] and against inflammatory diseases (antagonists) such as asthma.[37]

All of the adenosine receptor agonists synthesized thus far are structurally related to adenosine itself, in which the ribose moiety is mainly intact.[1] On the ribose, 5′-alkyluronamides groups are tolerated, and in some cases (*N*-methyl and *N*-ethyl) substitutions of such groups have been found to enhance potency at either A_{2A} or A_3 receptors. Positions on the structure of adenosine providing flexibility of substitution, in general for adenosine agonists, exist at the N^6- and C^2-positions. At the N^6-position most alkyl or aryl derivatives are A_1 selective, and at the C^2-position many C, N, or O derivatives are A_{2A} selective. N^6-Benzyl analogues also containing the 5′-uronamide modification, such as IB-MECA, have been found to be potent and selective A_3 agonists. At the N^6-position, only primary and secondary amino derivatives have thus far displayed substantial affinity at any of the adenosine receptors.[27] In this study we have shown that novel N^6-urea analogues (particularly *N*-phenylureas) have considerable affinity for the receptors. Features known to produce A_3 selectivity in the corresponding N^6-benzyladenosine

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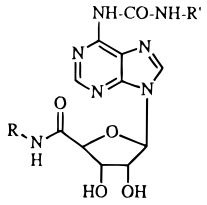
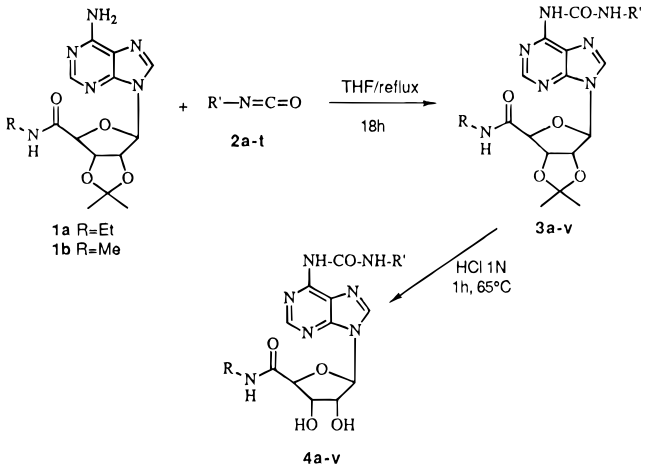
Table 1. Affinities of N^6-(Substituted-carbamoyl)adenosine-5′-uronamide Derivatives in Radioligand Binding Assays at Rat Brain A_1, A_{2A}, and A_3 Adenosine Receptors



			K_i (nM) or % inhibition				
compd	R	R′	A_1[a]	A_{2A}[b]	A_3[c]	A_1/A_3	A_{2A}/A_3
IB-MECA			54 ± 5	56 ± 8	1.1 ± 0.3	49	51
4a	Et	*t*-C_4H_9	1440 ± 59	3670 ± 140	1930 ± 570	0.75	1.9
4b	Et	*n*-C_8H_{17}	448 ± 60	3070 ± 310	326 ± 50	1.4	9.4
4c	Et	phenyl	110 ± 7.6	5364 ± 786	39 ± 16	2.8	140
4d	Et	2-CF_3-phenyl	384 ± 46	10000	54 ± 9.9	7.4	190
4e	Et	3-CF_3-phenyl	700 ± 100	2300 ± 400	77 ± 50	9.1	30
4f	Et	4-CF_3-phenyl	739 ± 49	4830 ± 840	150 ± 25	4.9	32
4g	Et	2-F-phenyl	77 ± 2.3	528 ± 35	34 ± 8.8	2.2	15
4h	Et	3-F-phenyl	103 ± 6.8	611 ± 102	39 ± 17.6	2.6	16
4i	Et	4-F-phenyl	85 ± 5.8	1384 ± 60	19 ± 6.1	4.4	72
4j	Et	2-MeO-phenyl	73 ± 5.7	1160 ± 58	30 ± 1.2	2.4	38
4k	Et	3-MeO-phenyl	113 ± 15	3856 ± 239	42 ± 30	2.7	92
4l	Et	4-MeO-phenyl	33 ± 2.0	3363 ± 503	6.6 ± 3.6	4.9	510
4m	Et	2-Cl-phenyl	111 ± 22	1337 ± 80	7.4 ± 6.1	15	180
4n	Et	3-Cl-phenyl	45 ± 3.9	420 ± 19	4.4 ± 1.5	10	96
4o	Et	4-Cl-phenyl	72 ± 8	1488 ± 99	114 ± 65	0.6	13
4p	Et	2-I-phenyl	179 ± 33	440 ± 60	32 ± 3.2	5.6	14
4q	Et	3-I-phenyl	16 ± 2.2	3939 ± 843	30 ± 8.5	0.5	130
4r	Et	4-I-phenyl	64 ± 3	561 ± 117	101 ± 24	0.6	5.6
4s	Et	4-Me-phenyl	124 ± 8.0	351 ± 45	32.3 ± 7.6	3.8	11
4t	Et	3-Br-phenyl	252 ± 25	286 ± 36	275 ± 74	0.9	1
4u	Me	3-Cl-phenyl	550 ± 81	6698 ± 136	115 ± 26	4.8	58
4v	Me	4-MeO-phenyl	491 ± 82	8747 ± 1821	56 ± 15.1	0.9	160

[a] Displacements of specific [^{3}H]CHA binding (A_1) in rat whole brain homogenates expressed as K_i ± SEM in nM (n = 3−4). [b] Displacements of specific [^{3}H]CGS 21680 binding (A_{2A}) in rat striatal homogenates expressed as K_i ± SEM in nM (n = 3−4). [c] Displacements of specific binding of [^{125}I]AB-MECA from membranes of CHO cells stably transfected with the rat A_3-cDNA, expressed as K_i ± SEM in nM (n = 3).

Scheme 1



derivatives[29] have been incorporated in the urea derivatives to test parallels in structure−activity relationships.

Results and Discussion

The preparation of the compounds **4a**−**v** was performed following the general synthetic strategy depicted in Scheme 1. For selective acylation of the N^6-amino group of adenosine, which has low chemical reactivity, it was necessary to protect the hydroxyl groups of the ribose moiety. Reaction of 2′,3′-isopropylidene-protected NECA or MECA (**1a,b**) with the appropriate isocyanate (**2a**−**t**) in the presence of a catalytic amount of triethylamine at reflux afforded the adducts **3a**−**v** in good yield. When not commercially available, the isocyanate was prepared by reacting the corresponding substituted anilines using trichloromethyl chloroformate, as described in the literature.[38]

Derivatives **3a**−**v** were deprotected in aqueous 1 N HCl and dioxane at 65 °C to furnish the desired N^6-substituted adenosineuronamides (**4a**−**v**) in a good yield.

The derivatives (**4a**−**v**) were tested in radioligand binding assays for affinity at rat brain A_1, A_{2A}, and A_3 adenosine receptors.

In previous studies[27,29] it has been demonstrated that combined modification of adenosine at the 5′- and N^6-positions significantly increases affinity for the A_3 receptor subtype with a different degree of selectivity. In the present study, we have synthesized several adenosine analogs containing both the N^6-alkyl- or -arylcarbamoyl groups. While N^6-alkylcarbamoyl derivatives **4a,b** had low affinity at A_1, A_{2A}, and A_3, adenosine receptor subtypes without significant selectivity, interesting results were obtained with the N^6-arylcarbamoyl derivatives (Table 1). Some of them, specifically the N^6-((2-chlorophenyl)carbamoyl)-, N^6-((3-chlorophenyl)carbamoyl)- and N^6-((4-methoxyphenyl)-carbamoyl)adenosine-5′-ethyluronamides (**4l**−**n**), were

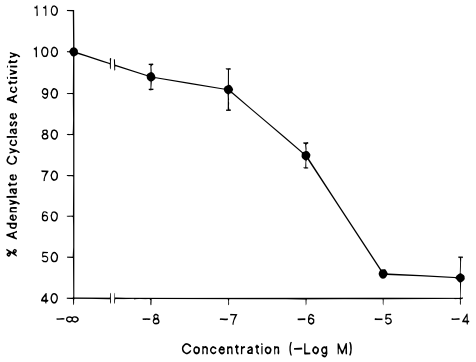


Figure 1. Inhibition of adenylate cyclase by the adenosine derivative N^6-((3-chlorophenyl)carbamoyl)adenosine-5′-ethyluronamide, **4n**, in membranes from CHO cell stably transfected with rat A_3 receptors. The assay was carried out as described in the Experimental Section in the presence of 1 μM forskolin. Each data point is shown as mean ± SEM for three determinations. The IC_{50} value was 0.85 ± 0.14 μM.

found to have affinity at A_3 receptors in the low nanomolar range.

Nevertheless, while a good separation exists between A_3 and A_{2A} receptor affinities (A_{2A}/A_3 ratio ranging from 96- to 510-fold), the A_3 vs A_1 selectivity appears to be moderate (from 5- to 15-fold). The 3-iodophenyl derivative **4q**, which is closest to the structure of IB-MECA, was found to be less potent (27-fold) than the reference compound and A_3 vs A_1 unselective.

The comparison among N^6-(arylcarbamoyl)adenosine-5′-ethyluronamide derivatives showed that both affinity and selectivity for the A_3 receptor subtype seems to correlate more with type of substitution on the phenyl ring than with the position of the substituent. Since the 5′-*N*-methyl substitution has been reported to generally increase the A_3 vs A_1 selectivity over the 5′-*N*-ethyl substitution,[29] we have introduced the *N*-methyl group in two (**4l,n**) of the most potent compounds of the *N*-ethyl series. In contrast with the expected results, based on the observation mentioned above, the compounds **4u,v** showed a decrease of both selectivity and affinity (Table 1).

One of the most potent derivative in binding assay, **4n**, was also found to be a full agonist in a specific functional model such as the inhibition of adenylate cyclase in membranes of CHO cells stably transfected with the rat A_3 receptor (Figure 1).

Compound **4n** showed an IC_{50} value of 0.85 ± 0.14 μM and a maximal inhibition of 54 ± 6% at the concentration of 10^{-4} M. Under the same conditions, 10^{-4} M IB-MECA caused an inhibition of adenylyl cyclase by 76.0 ± 3%. Thus, a basic amino group at the 6-position is not required for activation of A_3 receptors by adenosine derivatives. The IC_{50} value, 190-fold higher than the K_i value observed in binding experiments, is in agreement with previous study in which a factor of approximately 2 orders of magnitude between binding and functional data was found.[39]

Conclusions

The present study provides useful information to further investigate the structure−activity relationships of the A_3 adenosine receptor subtype. The most interesting and unexpected finding was that the amino group at the N^6-position of adenosine may be acylated to form a urea with retention of affinity at adenosine receptors and, in particular, at the A_3 receptor subtype. Some N^6-arylcarbamoyl derivatives (**4l−n**) had high affinity, but only moderate selectivity. However, due to variable species differences in ligand affinity at this receptor subtype,[32,33] it will be necessary to establish the selectivity of these novel A_3 agonists at human cloned adenosine receptors. Although the N^6-arylcarbamoyl derivatives do not possess a degree of selectivity as high as that achieved for both N^6-benzyladenosine[29] and 2-substituted N^6-benzyladenosine derivatives,[30] improvements may be obtained through the introduction of a variety of substituents on the aromatic ring present at N^6-position.

Experimental Section

Chemistry. Infrared spectra (IR) were measured on a Perkin-Elmer 257 instruments. ^{1}H NMR were determined in $CDCl_3$ or DMSO-d_6 solutions with a Bruker AC 200 spectrometer. Peaks positions are given in parts per million (δ) downfield from tetramethylsilane as internal standard, and *J* values are given in hertz. Petroleum ether refers to the fractions boiling at 40−60 °C. Melting points were determined on a Buchi-Tottoli instrument and are uncorrected. Chromatography was performed with Merck 60−200 mech silical gel. All products reported showed IR and ^{1}H NMR spectra in agreement with the assigned structures. Elemental analyses were performed by the microanalytical laboratory of the Department of Chemistry, University of Ferrara, and were within ±0.4% of the theoretical values for C, H, and N.

General Procedure for the Preparation of 2′,3′-*O*-Isopropylidene-N^6-(subsituted-carbamoyl)adenosine-5′-*N*-ethyluronamide (3a−t) and 2′,3′-*O*-Isopropylidene-N^6-(substituted-carbamoyl)adenosine-5′-*N*-methyluronamide (3u−v). 2′,3′-Isopropylidene-NECA or MECA **1a,b** (0.43 mmol) was dissolved in freshly distilled THF (4 mL), and the appropriate isocyanate **2a−t** (1.3 equiv) and a catalytic amount of triethylamine (two drops) were added. The mixture was refluxed under argon for 18 h. Then the solvent was removed under reduced pressure, and the residue was purified by flash chromatography (CH_2Cl_2−EtOAc 20%) to afford the desired compound **3a−v**.

The following spectral data are reported as example.

2′,3′-*O*-Isopropylidene-N^6-(*n*-octylcarbamoyl)adenosine-5′-*N*-ethyluronamide (3b): yield 70%; pale yellow oil; IR (neat, cm^{-1}) 3445, 1730, 1620, 1560; ^{1}H NMR ($CDCl_3$) δ 0.67 (t, 3H, *J* = 7), 0.88 (t, 3H, *J* = 6.8), 1.22−1.29 (m, 12H), 1.41 (s, 3H), 1.62 (s, 3H), 2.89−2.96 (m, 2H), 3.39 (q, 2H, *J* = 6.8), 4.72 (d, 1H, *J* = 2), 5.50−5.54 (m, 2H), 6.25 (d, 1H, *J* = 2), 6.64 (t, 1H, *J* = 2), 8.48 (s, 1H), 8.49 (s, 1H), 9.36 (bs, 1H), 9.52 (t, 1H, *J* = 2). Anal. ($C_{20}H_{29}N_7O_5$) C, H, N.

2′,3′-*O*-Isopropylidene-N^6-(phenylcarbamoyl)adenosine-5′-*N*-ethyluronamide (3c): yield 70%; white solid, mp 127−132 °C (CH_2Cl_2−Et_2O); IR (KBr, cm^{-1}) 3450, 1740, 1610, 1565, 1210; ^{1}H NMR ($CDCl_3$) δ 0.75 (t, 3H, *J* = 7), 1.42 (s, 3H), 1.63 (s, 3H), 2.96−3.03 (m, 2H), 4.74 (d, 1H, *J* = 1.8), 5.47−5.57 (m, 2H), 6.21 (d, 1H, *J* = 1.8), 6.52 (t, 1H, *J* = 2), 7.14−7.17 (m, 1H), 7.33−7.41 (m, 2H), 7.60−7.65 (m, 2H), 8.32 (s, 1H), 8.60 (s, 1H), 8.98 (bs, 1H), 11.72 (s, 1H). Anal. ($C_{22}H_{25}N_7O_5$) C, H, N.

General Procedure for the Preparation of N^6-(Substituted-carbamoyl)adenosine-5′-*N*-ethyluronamide (4a−t) and N^6-(Substituted-carbamoyl)adenosine-5′-*N*-methyluronamide (4u−v). A solution of isopropylidene derivative **3a−v** (0.084 mmol) in aqueous 1 N HCl (5 mL) and dioxane (5 mL) was stirred at 65 °C for 1 h. Then the solvents was removed under reduced pressure, and the residue was crystallized from ethanol to afford the desired compound **4a−v**.

The following spectral data are reported as example.

***N*^{6}-(*n*-Octylcarbamoyl)adenosine-5′-*N*-ethyluronamide (4b):** yield 60%; pale yellow solid; mp 177−179 °C (EtOH); IR (KBr, cm^{-1}) 3500−3100, 1675, 1600, 1320; ^{1}H NMR (DMSO-d_6) δ 0.76 (t, 3H, *J* = 6.8), 0.98 (t, 3H, *J* = 7), 1.15−1.42 (m, 12H), 3.08−3.14 (m, 4H), 4.12−4.14 (m, 1H), 4.30 (s,

1H), 4.55−4.57 (m, 1H), 3.80−4.30 (bs, 2H), 6.02 (d, 1H, J = 6), 8.42 (s, 1H), 8.54 (s, 1H), 8.82 (bs, 1H), 9.04 (bs, 1H), 10.10 (bs, 1H). Anal. ($C_{21}H_{32}N_7O_5$) C, H, N.

***N*^{6}-(Phenylcarbamoyl)adenosine-5′-*N*-ethyluronamide (4c):** yield 75%; white solid; mp 171−174 °C (EtOH); IR (KBr, cm^{-1}) 3500−3100, 1675, 1600, 1560, 1520, 1320; ^{1}H NMR (DMSO-d_6) δ 1.08 (t, 3H, J = 7), 3.15−3.23 (m, 2H), 4.21−4.23 (m, 1H), 4.36 (s, 1H), 4.64−4.48 (m, 1H), 5.67 (d, 1H, J = 6), 5.78 (d, 1H, J = 4), 6.10 (d, 1H, J = 5), 7.06−7.11 (m, 1H), 7.32−7.40 (m, 2H), 7.62−7.66 (m, 2H), 8.50 (t, 1H, J = 2), 8.73 (s, 1H), 8.80 (s, 1H), 10.27 (s, 1H), 11.78 (s, 1H). Anal. ($C_{19}H_{25}N_7O_5$) C, H, N.

Biological Studies: A_1, A_{2A}, and A_3 Receptor Binding Assays. The rat brain tissues (whole brain and striatum) were obtained from male Sprague−Dawley rats (Charles-River, Calco, Italy) weighing 150−200 g. Adenosine A_1 and A_{2A} receptor binding assays were performed according to Bruns et al.[11] and Jarvis et al.[18] using [^{3}H]-*N*^{6}-cyclohexyladenosine ([^{3}H]CHA and [^{3}H]-2-[[*p*-(2-carboxyethyl)phenethyl]amino]-5′-*N*-(ethylcarboxamido)adenosine ([^{3}H]CGS 21680), as radioligands, respectively. Both radioligands were purchased from NEN Research Products, (Boston, MA). Binding assay to Chinese hamster ovary (CHO) cells stably transfected with the rat brain A_3 receptor was performed as described by Olah et al.[33] using [^{125}I]-*N*^{6}-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]AB-MECA) as radioligand. The IC_{50} values were calculated by probit analysis based on at least six concentration of each compound. K_i values were calculated from the Cheng−Prusoff[40] equation using 1.0, 18.5, and 1.48 nM as K_d values in A_1, A_{2A}, and A_3 binding assay, respectively.

Adenylate Cyclase Assay. Adenylyl cyclase was assayed in membranes from CHO cells stably expressing the rat A_3 receptor, prepared as above, using a previously reported method.[27,35] The method involved addition of [α-^{32}P]ATP to membranes in the presence of 1 μM forskolin to stimulate adenylyl cyclase and 100 μM papaverine as a phosphodiesterase inhibitor. Membranes were suspended in 75 mM Tris, 200 mM NaCl, 1.25 mM $MgCl_2$, pH 8.12, at 4 °C (TNM buffer) to give a final concentration of 0.1 mg/mL, and 2 units/mL adenosine deaminase was added. Adenylyl cyclase assays consisted of 40 μL of membrane suspension, 40 μL of cyclase mixture (TNM buffer supplemented with 140 μM dATP, 5 μM GTP, 30 units/mL creatine kinase, 5 mM creatine phosphate, 2.2 mM dithiothreitol, 100 μM papaverine, and 1.5 μCi of [α-^{32}P]ATP), and 20 μL of test compounds (**4n**). Assays were conducted at 30 °C for 15 min and terminated by addition of a stop solution containing 20 000 cpm/mL ^{3}H-labeled cyclic AMP. The total radiolabeled cyclic AMP was isolated on columns of Dowex 50 ion exchange resin and alumina, and quantities were determined by liquid scintillation counting. Maximal inhibition of adenylate cyclase activity corresponds to ∼40% of total activity under conditions of stimulation (typically by 6−8-fold) in the presence of 1 μM forskolin. IC_{50} values were calculated using InPlot (GraphPad, San Diego, CA).

Abbreviations: [^{125}I]AB-MECA, [^{125}I]-*N*^{6}-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide; CGS 21680, [[2-[4-(2-carboxyethyl)phenyl]ethyl]amino]-5′-*N*-(ethylcarboxamido)adenosine; CHA, (*R*)-*N*^{6}-cyclohexyladenosine; DMSO, dimethylsulfoxide; IB-MECA, *N*^{6}-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; MECA, 5′-*N*-methylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; THF, tetrahydrofuran; Tris, tris(hydroxymethyl)aminomethane.

Supporting Information Available: Experimental data for **3a**−**v** and **4a**−**v** (11 pages). Ordering information is given on any current masthead page.

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