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A SELECTIVE AGONIST AFFINITY LABEL FOR A_3 ADENOSINE RECEPTORS

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A newly synthesized, chemically reactive adenosine derivative, N^6-(3-isothiocyanatobenzyl)adenosine-5'-N-methyluronamide, was found to bind selectively to A_3 receptors. K_i values for this isothiocyanate derivative in competition binding at rat brain A_1, A_{2a}, and A_3 receptors were 145, 272 and 10.0 nM, respectively. A preincubation with this derivative resulted in irreversible inhibition of radioligand binding at rat A_3 receptors in membranes of transfected CHO cells or RBL-2H3 mast cells, but not at rat A_1 or A_{2a} receptors. The loss of binding sites for 0.1 nM [^{125}I]N^6-(4-aminobenzyl)adenosine-5'-N-methyluronamide, a high affinity A_3 receptor radioligand, in transfected CHO cell membranes was concentration-dependent with an IC_{50} of 50 nM. No change was observed in the K_d value of the remaining A_3 receptor sites. The inhibition was also insensitive to theophylline (1 mM), consistent with the pharmacology of rat A_3 receptors. Structurally similar adenosine analogues lacking the chemically reactive isothiocyanate group failed to irreversibly inhibit A_3-binding.

Adenosine agonists such as NECA (5'-N-ethylcarboxamidoadenosine, **1**, Figure 1) are known to facilitate the release of inflammatory mediators from mast cells (1,2). The action occurs via activation of phospholipase C and is accompanied by a rise in intracellular calcium concentration. Recently, a molecular explanation was offered for this unique action of adenosine, *i.e.* mast cells express the novel A_3 adenosine receptor (3), which was discovered through cloning from a rat brain cDNA library (4). Xanthines that act as antagonists at A_1 and A_2 adenosine receptors do not antagonize the response (4,5). For example, the xanthine amine congener, which has nanomolar affinity at rat brain A_1 receptors, binds to rat brain A_3 receptors with a K_i value of 29 μM

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Abbreviations used: [^{125}I]AB-MECA, N^6-(4-amino-3-iodobenzyl)adenosine-5'-N-methyluronamide; CGS 21680, 2-(carboxyethylphenylethylamino)adenosine-5'-carboxamide; CHO, Chinese hamster ovary; IB-MECA, N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide; PIA, *R*-N^6-phenylisopropyl-adenosine; Tris, tris(hydroxymethyl)aminomethane.

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(6) and does not antagonize functional effects (inhibition of adenylate cyclase) of activation of rat A_3 receptors (4).

A_3 adenosine receptors have been proposed as a therapuetic target in inflammatory diseases (7-9) and in diseases of the central nervous system (10,11). Adenosine is known to be a bronchoconstrictor in asthmatic patients (8,9), although the mechanism of this effects is currently unclear, since until recently selective probes have not been available. A selective A_3 antagonist might be useful as an anti-inflammatory drug (7). Chronic activation of A_3 receptors has also been proposed in treating cerebral ischemia (11).

In this study we have designed a chemically-reactive receptor probe, an adenosine derivative bearing an isothiocyanate group, **4** (Figure 1), and have shown that it is selective for A_3 receptors in models of reversible and irreversible binding in membranes from CHO cells stably transfected with rat brain A_3 receptors and in RBL-2H3 cell membranes.

MATERIALS AND METHODS

Synthesis:

[^{3}H]CGS 21680 was obtained from Dupont NEN (Boston, MA) and [^{125}I]AB-MECA was prepared as described (12). Compound **3** was prepared as described (13).

N^6-(3-Isothiocyanatobenzyl)adenosine-5'-N-methyluronamide (**4**)

N^6-(3-Aminobenzyl)adenosine-5'-N-methyluronamide (**3**, 14.1 mg, 35.3 μmol) was partially dissolved in dry chloroform (6 mL), and saturated sodium bicarbonate solution (2 mL) was added. Thiophosgene (16 μL, 210 μmol) was added at once with vigorous stirring. After 5 min. the reaction was complete (silica TLC, Rf 0.79, chloroform:methanol:ammonium hydroxide 80:20:1), and more chloroform (20 ml) and more water (5 mL) were added to break the emulsion. The phases were separated, and the aqueous phase was washed with chloroform (3 x 25 mL). The organic phases were combined, dried ($MgSO_4$) and the solvent was removed under vacuum to obtain 8 mg (51 % yield) of the pure compound. Mp: 170-174 °C decomp.

MS EI: 441 (M^+), 325, 311, 282. IR (NaBr) 2051.3, 2103.5 cm^{-1}.

^{1}H NMR DMSO-d_6 2.70 (d, J = 4.6 Hz, 3H, NH*CH3*) , 4.14 (dd, J = 4.5 Hz J = 1.1 Hz, 1H, H-3'), 4.31 (d, J = 1.2, 1H, H-4'), 4.59 (dd, J = 7.3 Hz J = 4.7 Hz, 1H, H-2'), 4.70 (br s, 2H, N^6-*CH2*Ph), 5.97 (d, J = 7.6 Hz, 1H, H-1'), 7.24-7.31 (m, 1H), 7.31-7.42 (m, 3H), 8.29 (s, 1H, H-2), 8.44 (s, 1H, H-8),8.58 (br s, 1H, N^6*H*-CH$_2$Ph), 8.80-8.87 (m, 1H, N*H*CH$_3$).

Biochemical assays:

Preparation of membranes for binding assays:

CHO cells stably transfected with cDNA for rat A_3 receptors and RBL-2H3 cells were grown by methods described previously (5,12). Membranes from CHO cells, from RBL-2H3 cells were homogenized in ice cold 50 mM Tris HCl / 10 mM $MgCl_2$ /1 mM EDTA (pH 8.25, "50/10/1") using a Polytron (Kinematica Gmbh, Luzern, Switzerland) at a setting of 6 for 10 sec. Rat forebrain (for A_1 binding) or striatum (for A_{2a} binding) were homogenized in ice cold 50 mM Tris HCl (pH 7.4). The membrane suspension was then centrifuged at 37,000 X g for 10 min at 4°C. The pellet was resuspended (20 mg tissue/ml) in the above buffer solution, preincubated at 30 °C for 30 min with 3 IU/ml of adenosine deaminase, and the membranes were again homogenized and centrifuged. Finally the pellet was suspended in buffer (100 mg wet weight per ml) and stored at

-70°C. Protein was determined using the BCA protein assay reagents (Pierce Chemical Co., Rockford, IL).

Radioligand binding:

For all binding experiments, adenosine deaminase was present (3 IU/ml) during the incubation with radioligand. [^{3}H]CGS 21680 binding to striatal A_{2a}-receptors in rat brain was carried out as described (14) using 20 μM 2-chloroadenosine to determine non-specific binding. The binding of [^{3}H]R-PIA to rat cortical A_1-receptors was carried out by the method described (5).

The binding of [^{125}I]AB-MECA to A_3-receptors membranes from transfected CHO cells and RBL-2H3 cells was carried out as described (12). Unlike our previous study of A_3 receptors in brain membranes (6), a xanthine (A_1/A_{2a} antagonist) was not added to prevent non-selective binding of the radioligand, since the background level of A_1/A_{2a} receptors was minimal. A_1 receptor binding of 0.41±0.38 and 3.3±0.5 fmol/mg protein was found in CHO and RBL cell membranes, respectively, using 1.0 nM [^{3}H]PIA. Similarly, A_{2a} receptor binding of 2.8±2.0 and 26±3 fmol/mg protein was detected using 5.0 nM [^{3}H]CGS 21680.

For competition studies, IC_{50} values were determined using the Inplot computer program (Graphpad, San Diego, CA) and converted to apparent K_i values using K_D values and the Cheng-Prusoff equation (15). K_D values for [^{125}I]AB-MECA binding to A_3 receptors in transfected CHO cells and in RBL-2H3 cells were 1.48 and 3.61 nM, respectively (12). K_D values in rat brain for [^{3}H]PIA and [^{3}H]CGS 21680 binding were 1.0 and 15 nM, respectively (5,14), at A_1 and A_{2a} receptors. Concentrations of [^{125}I]AB-MECA, [^{3}H]PIA, and [^{3}H]CGS 21680 used in competition experiments were 0.1, 1.0, and 5.0 nM, respectively.

Preincubation of membranes with inhibitor:

Membranes were incubated with adenosine derivatives in pH 8.25 50/10/1 buffer containing adenosine deaminase, for 1 h at 25 °C, subjected to three washing cycles, consisting of centrifugation at 37,000 X g and resuspended of the pellet in 50/10/1 buffer prior to radioligand binding. For theophylline exposure experiments, membranes were preincubated with theophylline at 25°C for 30 min, and then **4** was added immediately for an additional incubation at 25°C for 60 min. At the end of this sequence, the membranes were washed by repeated centrifugation and resuspension and subjected to binding with the appropriate radioligand. At the final step, prior to radioligand binding, the membranes were homogenized manually using a glass tissue grinder.

RESULTS AND DISCUSSION

The structure activity relationships of N^6 and 5'-substituted adenosine derivatives at rat A_3 adenosine receptors have been elucidated (5,13). The combination of the 5'-N-methyluronamide modification and a 3-substituted N^6-benzyl substituent was found to generally result in moderate selectivity as agonists for A_3 receptors. One such derivative, IB-MECA, **2**, which was 50-fold A_3-selective in binding assays, was found to selectively activate A_3 receptors also *in vivo* (10). Thus, the 3-position of the benzyl ring of this analogue was chosen as the site for incorporation of a chemically reactive functional group. The electrophilic group selected for this study was the isothiocyanate group, which was shown previously to serve as a cross-linking point for covalent reaction of ligands selective for A_1 and A_{2a} subtypes of adenosine receptors (16-18).

An N^6-(3-aminobenzyl) derivative of adenosine-5'-N-methylcarboxamide (13), **3**, was converted to the corresponding isothiocyanate derivative, **4**, by treatment with

thiophosgene (Figure 1). This conversion was carried out without protection of the 2'- and 3'-hydroxyl groups.

Compound **4** was then characterized in adenosine receptor binding assays. Competition of binding of [^{125}I]AB-MECA, a high affinity A_3-agonist (12), in membranes from CHO cells stably transfected with rat brain A_3-receptors was measured under "reversible" conditions. The apparent K_i value of **4** at A_3 receptors was found to be 10.0±2.3 nM (n = 3), comparable to the affinity of the selective agonist **2** and the precursor **3** (13). At A_1-receptors in rat cortex, a K_i value of 145±41 nM (n = 3) for inhibition of [^{3}H]PIA was determined. Thus, the selectivity ratio of **4** for A_3- versus A_1-receptors in the rat, based on K_i values, was 14-fold. At A_{2a}-receptors in rat striatum, the K_i value in displacement of the A_{2a}-selective agonist [^{3}H]CGS21680 was 272±93 nM (n = 3), indicating a 27-fold selectivity ratio in binding of **4** at A_3- vs. A_{2a}-receptors in the rat brain. The Hill coefficients for displacement of binding at rat brain A_1-, A_{2a}-, or A_3-receptors were approximately equal to 1.

Competition by **4** of binding of [^{125}I]AB-MECA, in RBL-2H3 cell membranes was also measured under "reversible" conditions. An apparent K_i value of 23.0±7.8 nM (n = 3) was determined. Non-specific binding in control and treated RBL-2H3 cell membranes was nearly identical and amounted to 15-20% of total binding at 0.1 nM [^{125}I]AB-MECA.

At higher concentrations, compound **4** was found to significantly inhibit radioligand binding at A_3-receptors in an irreversible manner, in both transfected CHO cells and RBL-2H3 cells (Figure 2). Preincubation of transfected CHO cell membranes with **4** caused a dose-dependent, irreversible antagonism of the specific binding of 0.1 nM [^{125}I]AB-MECA, with an IC_{50} value of approximately 50 nM. This IC_{50} value was 4-times greater than the IC_{50} value in competitive displacement of [^{125}I]AB-MECA in the same membranes. The IC_{50} value for irreversible inhibition of A_3 receptors in RBL-2H3 cell membranes was approximately 400 nM. The irreversible nature of inhibition by the isothiocyanate derivative was demonstrated by the failure of repeated washing to

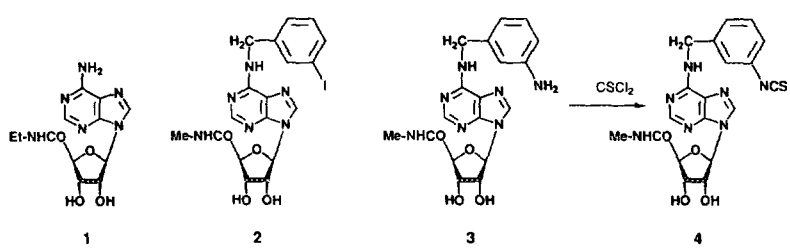

Figure 1. Structures and synthesis of adenosine derivatives having high affinity and/or selectivity as A_3 receptor agonists. NECA, **1**, has K_i values of 6.3, 10.3, and 113 nM at rat A_1-, A_{2a}-, and A_3-receptors, respectively (5). The 3-amino derivative (13), **3**, was converted to the electrophilic isothiocyanate analogue, **4**, using thiophosgene. The 3-amino derivative, **3**, is also selective for A_3-receptors (36-fold and 28-fold vs. A_1 and A_{2a} receptors, respectively), but is not chemically reactive in receptor binding.

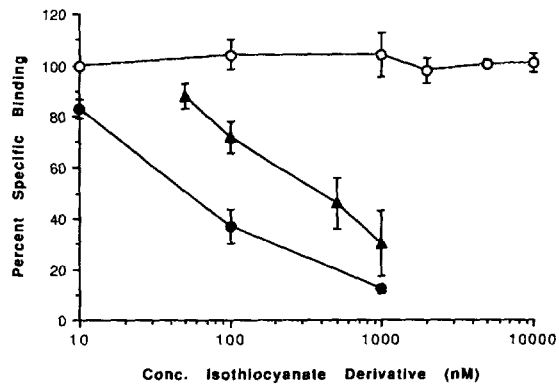

Figure 2. Dose-dependent inhibition by **4** of radioligand binding at A_3-adenosine receptors in: A_3 receptors in stably transfected CHO cell membranes (filled circles, n = 2-4) and A_3 receptors in RBL-2H3 cell membranes (triangles, n = 3), and A_1 receptors in rat brain cell membranes (open circles, n = 3-4). The preincubation with **4** or control was carried out for 1 h at 25°C, and the subsequent binding assay involved a 60 min at 37°C incubation followed by rapid filtration. Concentrations of [^{125}I]AB-MECA and [^{3}H]PIA used in competition experiments were 0.1, 1.0, and 5.0 nM, respectively. Each data point represents the mean±S.E.M of three separate determinations, each done in duplicate.

regenerate the A_3-receptor binding site. At A_1-adenosine receptors in rat cortical membranes, **4** was not effective as an irreversible inhibitor (Figure 2), even at a concentration 70X the K_i value. Following preincubation with **4** at 1 μM the percentage of [^{3}H]PIA binding sites was found to be 104±9% (n = 4) of specific control binding (membranes incubated in parallel) versus 12% (n = 2) of specific control binding [^{125}I]AB-MECA binding in the CHO cells.

As a control experiment for demonstrating that irreversible binding depends on the presence of the isothiocyanate group, CHO cell membranes were pre-incubated with compounds **2** and **3**, which are not chemically reactive due to the absence of an electrophilic group. The K_i values for these compounds at rat A_3 receptors were found to be 1.1 and 16 nM, respectively (13). The [^{125}I]AB-MECA binding sites in these membranes were nearly completely restored following the same washing procedure that was ineffective in regenerating the binding following incubation with **4**. Thus following a preincubation with compound **2** (10 nM) or **3** (100 nM), 98 or 90% (n = 2), respectively, of the specific A_3 binding was regenerated.

Binding of [^{125}I]AB-MECA to A_3 receptors in CHO cell membranes exposured to **4** was saturable (Figure 3) and of the same affinity as in control membranes, but the B_{max} was diminished. A preincubation with 100 nM **4** followed by washing resulted in a K_d value at the remaining sites of 1.63±0.22 nM, compared to 1.82±0.45 nM (n = 4) in the control membranes. The B_{max} values in treated and control membranes were 1.36±0.05 and 2.30±0.12 pmol/mg protein, respectively, representing a loss in the density of binding sites of 41%. The loss of binding sites determined by Scatchard

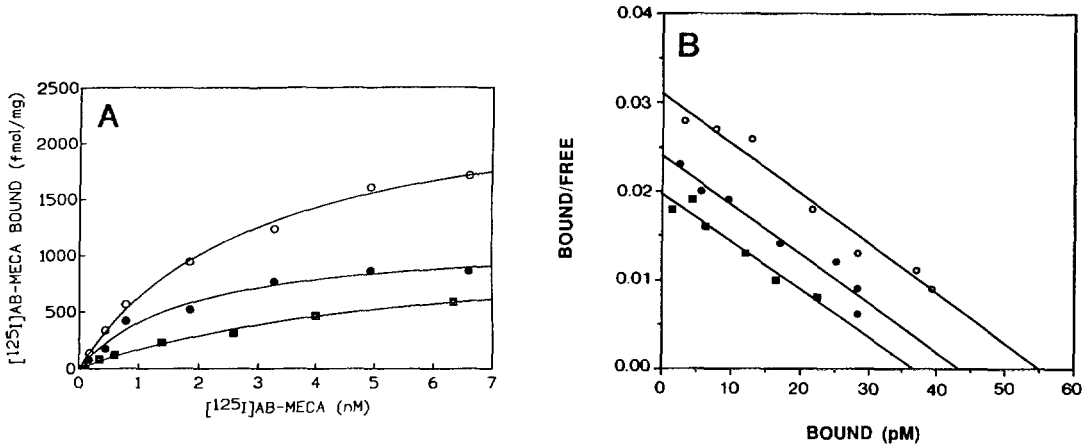

Figure 3. Saturation curve (A) and Scatchard transformation (B) for the binding of [^{125}I]AB-MECA to A_3-adenosine receptors in CHO cell membranes following a preincubation with compound **4** at 25°C for 1 hr. Specific binding for control membranes (open circles), and following exposure to compound **4** at 0.1 μM (filled circles) and 1.0 μM (squares) are shown. After three cycles of washing, the membranes were incubated with radioligand at 37°C for 60 min. The volume of incubation for radioligand binding (20-40 μg protein/tube) was 0.2 ml.

analysis was also concentration dependent. Following a preincubation with 1000 nM **4**, K_d and B_{max} values of 2.32 nM and 0.83 pmol/mg were determined.

The degree of ireversible inhibition of binding of [^{125}I]AB-MECA at rat brain A_3-receptors by **4** (at either 0.1 or 1.0 μM) was not affected by co-incubation with the non-selective A_1 and A_2 adenosine receptor antagonist theophylline (1 mM). This is consistent with the inability of theophylline to block rat A_3 receptor-mediated inhibition of adenylate cyclase (5).

Although most chemical affinity labels for receptors are antagonist-derived, a few irreversibly binding agonists have been reported. An A_{2a}-selective isothiocyanate derivative, *p*-DITC-APEC, effected the prolonged and irreversible activation of A_{2a} adenosine receptors in the guinea pig coronary artery (17). An A_1-selective isothiocyanate derivative, *m*-DITC-ADAC, was found to be an irreversible, full agonist in prolonging the S-H interval in guinea pig isolated hearts (18). An agonist-derived chemical affinity labels for adrenergic receptors has proven useful in pharmacological studies of cross-talk with adenosine receptors (19). Agonist affinity labels are also potentially of use in studying spare receptors, receptor turnover, affinity states, peptide mapping of the binding site, and receptor activation and desensitization (20).

Indeed we have established that the isothiocyanate derivative, **4**, binds irreversibly and selectively to A_3 receptors in transfected CHO cell membranes and in membranes from rat mast cells. Thus, we have introduced a new tool for the study of A_3 receptors, their molecular structure, regulation, and physiological role in the inflammatory (1-3), pulmonary (8,9), cardiovascular (21), and central nervous systems (10,11). It will be necessary to examine the functional effects (3-5) of this

isothiocyanate derivative as a function of time in order to establish whether it acts as a full agonist in functional assays and whether this action is maintained or rapid desensitization occurs.

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