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Agonist Derived Molecular Probes for A_{2A} Adenosine Receptors

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Abstract

The adenosine agonist 2-(4-(2-carboxyethyl)phenylethylamino)-5'-N-ethylcarboxamidoadenosine (CGS21680) was recently reported to be selective for the A_{2A} adenosine receptor subtype, which mediates its hypotensive action. To investigate structureactivity relationships at a distal site, CGS21680 was derivatized using a functionalized congener approach. The carboxylic group of CGS21680 has been esterified to form a methyl ester, which was then treated with ethylenediamine to produce an amine congener. The amine congener was an intermediate for acylation reactions, in which the reactive acyl species contained a reported group, or the precursor for such. For radioiodination, derivatives of p-hydroxyphenylpropionic, 2-thiophenylacetic, and p-aminophenylacetic acids were prepared. The latter derivative (PAPA-APEC) was iodinated electrophilically using [¹²⁵I]iodide resulting in a radioligand which was used for studies of

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competition of binding to striatal A₁ adenosine receptors in bovine brain. A biotin conjugate and an aryl sulfonate were at least 350-fold selective for A₁ receptors. For spectroscopic detection, a derivative of the stable free radical tetramethyl-1-piperidinyloxy (TEMPO) was prepared. For irreversible inhibition of receptors, *meta*- and *para*-phenylenediisothiocyanate groups were incorporated in the analogs. We have demonstrated that binding at A_{2A} receptors is relatively insensitive to distal structural changes at the 2-position, and we report high affinity molecular probes for receptor characterization by radioactive, spectroscopic and affinity labelling methodology.

INTRODUCTION

Adenosine acts as a modulator of activity in the cardiovascular system, nervous system, immune system, and other physiological systems (Williams, 1987). Adenosine receptors are subdivided into two subclasses, the A₁ and A₂ receptors, which are in general inhibitory and stimulatory, respectively, towards adenylate cyclase (Londos *et al.*, 1980). A₁ receptors in brain and adipocytes have been characterized through photoaffinity (Stiles *et al.*, 1985; Stiles *et al.*, 1986; Linden *et al.*, 1988; Stiles *et al.*, 1987), and chemical affinity labelling (Stiles *et al.*, 1988; Jacobson *et al.*, 1989a) and through enzymatic digests (Stiles, 1986), as a glycoprotein of molecular weight 38 000–40 000.

A_{2A} Adenosine receptors mediate the following physiological effects of adenosine: inhibition of platelet aggregation (Lohse *et al.*, 1988), immunosuppression (Polmar *et al.*, 1988), vasodilation (Olsson *et al.*, 1986) and antipsychotic-like action (Bridges *et al.*, 1987). Few selective agonists (Bridges *et al.*, 1988; Bruns *et al.*, 1986) or antagonists (Shamim *et al.*, 1989) for A_{2A} adenosine receptors are known. Recently an adenosine agonist, CGS21680, 2-(4-(2-carboxyethyl)phenylethylamino)-5'-N-ethylcarboxamidoadenosine, **1**, was reported to be 170-fold A_{2A} selective in binding assays (Jarvis *et al.*, 1989), and the only adenosine agonist found to be A_{2A} selective *in vivo* in cardiovascular models (Hutchison *et al.*, 1989). [³H]CGS21680 has been synthesized and utilized as a radioligand displaying 80–90% specific binding at A_{2A} adenosine receptors in rat striatum (Jarvis *et al.*, 1989).

In this study, structural modifications of CGS21680 are made at a distal site, using a functionalized congener approach. By this approach, analogs containing chains terminating structurally in chemical function groups are synthesized. An alkyl diamino spacer chain serves as a general site for derivatization, with retention of high affinity for A_{2A} adenosine receptors.

MATERIALS AND METHODS

CGS21680, **1**, (Hutchison *et al.*, 1989), and the methyl ester derivative, **2** (prepared by the action of diazomethane on CGS21680), were synthesized by one of the authors (A.J.H.). 1,3-phenylenediisothiocyanate (Stiles *et al.*, 1988) was prepared as described. New compounds were characterized (and resonances assigned) by 300 MHz proton nuclear magnetic resonance spectroscopy using a Varian XL-300 FT-NMR spectrometer (Varian Associates Inc., Sunnyvale, CA, USA). Unless noted, chemical shifts are expressed as ppm downfield from tetramethylsilane. Synthetic intermediates were characterized by NMR and by chemical ionization mass spectroscopy (CIMS, NH₃) using a Finnigan 1015 mass spectrometer modified with Extrel electronics or on a Finnigan 4500 MS (Finnigan Corp., San Jose, CA, USA). Adenosine analogs were characterized additionally by plasma desorption mass spectroscopy (Jacobson *et al.*, 1986), and were identified by the presence of positive ion peaks observed at *m* + 23. C, H, and N analysis was carried out by Atlantic

Microlabs (Norcross, GA, USA) ($\pm 0.4\%$ acceptable). N⁶-cyclopentyladenosine, XAC and ADAC were obtained from Research Biochemicals Inc., Natick, MA, USA. The 2-alkylamino-5'-carboxamidoadenosine derivatives were generally soluble in methanol, or mixtures of alcohol and acetonitrile, and were insoluble in ether or pure acetonitrile.

Synthesis of derivatives

Synthesis of 2-(4-(2-(2-aminoethylaminocarbonyl)ethyl)phenylethylamino)-5'-N-ethylcarboxamidoadenosine, 3, APEC—Compound **2** (100 mg, 0.195 mmol) was dissolved in ethylenediamine and heated at 50° C for 12 h. The solution was evaporated under a stream of nitrogen. Methanol and ether were added to give an oily precipitate, which solidified *in vacuo*. The solid was dissolved in methanol and reprecipitated with ether. The amorphous product (yield 96 mg, 91%) melted at 113–117° C. Characteristic ¹H NMR resonances in d₆-DMSO occurred at δ 8.02 (s, 1H, aromatic, C-8); 7.75 (m, 1H, NH γ to 1° amine); 7.11 (m, 4H, phenyl ring); 5.83 (d, 1H, J = 6.3 Hz, ribose C_{5'}); 4.71, 4.24, and 4.17, (each 1H, ribose); 3.00 (m, 2H, CH₂, p to 1° amine); 2.7 (m, 4H, α to phenyl ring); 2.33 (t, 2H, J = 7 Hz, CH₂, 3 to CO); and 0.96 (t, J = 7 Hz, CH₃).

Synthesis of 2-(4-(2-(2-(4-aminophenyl)methylcarbonyl)amino)-ethylaminocarbonyl)ethyl)phenylethylamino)-5'-N-ethylcarboxamidoadenosine, 6—Compound **7** (4.0 mg, 4.9 μ mol) and 5% palladium on 3 mg charcoal (Engelhard, Edison, NJ, USA) were added to 0.3 mL of an equivolume mixture of methanol, dimethylformamide, and acetic acid. The mixture was hydrogenated at 40 lb/in² for 6 h. The catalyst was removed by centrifugation. The title compound was isolated in 68% yield as a white solid (2.3 mg). An additional purification, by preparative TLC (silica, chloroform + methanol + acetic acid, 70: 25: 5 v/v) was necessary.

Radioiodination of 2-(4-(2-(2-(4-aminophenyl)methylcarbonyl)amino)-ethylaminocarbonyl)ethyl)phenylethylamino)-5'-N-ethylcarboxamidoadenosine—Ten μ L of a solution of compound **6** (0.1 mg/mL) in methanol was placed in a microcentrifuge tube and dried completely under a stream of nitrogen. The residue was dissolved in 30 μ L of 0.5 M sodium phosphate (pH 7.39, and mixed well with 1.5 mCi of [¹²⁵I]Na. The reaction was initiated by the addition of 10 μ L of aqueous chloramine T (1 mg/mL), and the entire reaction mixture was mixed by pipette aspiration for 4 min. The reaction was terminated by the addition of 10 μ L of aqueous sodium metabisulfite (2 mg/mL).

The product, [¹²⁵I]PAPA-APEC, was purified by (Milford, MA, USA) HPLC apparatus utilizing a C₁₈, μ Bondapak column, and a mobile phase consisting of methanol and 20 mM ammonium formate at pH 8.1. A shallow concave gradient pattern (curve No. 8 on Waters model 680 Automated Gradient Controller) was used. The percent methanol was varied from 60% at zero time to 50% after IOMin, with the remainder being ammonium formate solution. The flow rate was 1.0 mL/min, and the UV detector (254 nm) was set on the 0.1 absorbance unit scale. Four major absorbance peaks (Fig. 1) were detected at retention times of approximately 4 (two peaks), 5 (peak A), and 7 (peak B) minutes after injection. Unreacted compound **6** (peak A) and [¹²⁵I]PAPA-APEC (peak B), were identified by TLC on silica plates, in which the mobile phase consisted of a chloroform + methanol + acetic acid mixture in the ratio 85: 105 v/v/v. The R_f values of compound **6** and the product were 0.11 and 0.23, respectively. Peak B was the only radioactive fraction to display specific binding and the appropriate A_{2A} receptor pharmacology. A small radioactive contaminant was seen at approximately 6.5 min, but was distinguished from [¹²⁵I]PAPA-APEC by a R_f value of 0.36.

Synthesis of 2-(4-(2-(2-((4-(benzyloxycarbonylamino)phenyl)-methylcarbonylamino)ethylaminocarbonyl)ethyl)-phenylethylamino)-5'-N-ethylcarboxamidoadenosine, 7—2-(4-(2-Carboxyethyl)phenyl)ethylamino-5'-N-ethylcarboxamidoadenosine sodium salt (CGS21680C), 7.2 mg, 13 μ mol) was suspended in 0.5 mL of dimethylformamide and treated with 1-hydroxybenzotriazole (20 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (13 mg, 68 μ mol). The mixture was stirred for several min and then treated with 2-((4-(benzyloxycarbonylamino)phenylacetyl)amino)ethylamine (compound **18**, 5.5 mg, 17 μ mol). After 24 h, 4 mL of water was added and a precipitate was separated by centrifugation, washed with water, and dried at 50° C *in vacuo*. The title compound was obtained in 65% yield (7.0 mg), and was shown to be homogeneous by TLC (R_f = 0.18 on silica, chloroform + methanol + acetic acid, 85:10:5 v/v). The 300 MHz ^{1} H NMR spectrum was consistent with the assigned structure.

Representative acylation of APEC (used to synthesize compounds 4, 5, 9, 10–13, and 15)—APEC (3, 10 μ mol) was dissolved in 0.5 mL of dimethylformamide or in a 1:1 v/v mixture of isopropanol + acetonitrile. To form amides, an active ester (20 μ mol) was added. To form thioureas (compounds **11–13**, and **15**), the appropriate isothiocyanate derivative (20 μ mol, or 50 μ mol if bifunctional), was added. The reaction was followed by TLC, and generally was complete within several min. The solvent was evaporated under a stream of nitrogen. Acetonitrile and ether were added causing the product to precipitate. The product was recrystallized sequentially from DMF + acetonitrile + ether and from methanol + ether.

Synthesis of N-benzyloxycarbonyl derivatives—Methyl 4-aminophenylacetate hydrochloride (1.01 g, 5.0 mmol) was acylated using carbobenzoxy chloride (0.72 mL, 5.0 mmol) in a methanol + aqueous sodium bicarbonate mixture. The product, methyl 4-(benzyloxycarbonylamino) phenylacetate, **17**, was extracted into methylene chloride and isolated as an oil in quantitative yield. m-Aminobenzoic acid (2.94 g, 21 mmol) was suspended in 50 mL of methanol and treated with carbobenzoxy chloride (3.0 mL, 21 mmol). The mixture was sonicated for 10 min and water was added, resulting in the crystallization of the product, 3-(benzyloxycarbonylamino)benzoic acid, **19** (m.p. 218–219° C), obtained in 67% yield. C, H, and N analysis.

Synthesis of 2-(4(benzyloxycarbonylamino)phenylacetyl)amino-ethylamine, 18—Methyl 4-(benzyloxycarbonylamino)phenyl acetate (compound **17**, 1.3 g, 4.3 mmol) was treated with ethylenediamine (4 mL) and heated at 50° C for 10 min. Several cycles of evaporation under vacuum and addition of methanol, left an oil which could be crystallized from methanol/ether. Yield 0.80 g (56% yield), m.p. 186–190° C. C, H, and N analysis was correct for the 3/2 hydrate.

Synthesis of N-succinimidyl 3-(benzyloxycarbonylamino)benzoate, 20—Compound **19** (3-(benzyloxycarbonylamino)-benzoic acid, 0.30 g, 1.1 mmol), N-hydroxysuccinimide (0.30 g, 2.6 mmol), and EDAC (0.36 g, 1.9 mmol) were combined in 10 mL DMF. After being stirred for 12 h, the reaction mixture was treated with water and ethyl acetate. The organic layer was separated, washed (0.1 M HCl / 1 M bicarbonate), and evaporated leaving the product (m.p. 110–115° C) in 89% yield. C, H, and N analysis.

Biochemical assays: Radioligands ($[^3\text{H}]$ P1A, ($[^3\text{H}]$ NECA, and ($[^3\text{H}]$ CGS21680) were obtained from DuPont NEN Products Boston, MA, USA. Stock solutions of adenosine derivatives in the millimolar concentration range in dimethylsulfoxide were prepared for binding assays. The solutions were diluted as necessary and were stable to storage in the frozen state. For each assay at A_1 or at A_{2A} receptors, inhibition of binding by a range of

concentrations of xanthines was assessed in triplicate in at least three separate experiments. Protein was determined using the BCA (based on the complex with cuprous ions and bicinchoninic acid) protein assay reagents purchased from Pierce Chemical Co., Rockford, IL, USA. Increase in cyclic AMP in human platelets was measured using the method described in Newman *et al.* (1978).

Competitive binding assay in rat brain using [³H]PIA, [³H]CGS21680, and [³H]NECA—Inhibition of binding of 1 nM [³H]N⁶-phenylisopropyladenosine (specific activity 42.5 Ci/mmol), to A₁ adenosine receptors in rat cerebral cortex membranes, was assayed as described in Jacobson *et al.* (1987). Inhibition of binding by a range of concentrations of an adenosine derivative was assessed in triplicate in at least three separate experiments. At least seven different concentrations spanning three orders of magnitude (adjusted appropriately for the IC₅₀ of each compound), were used. IC₅₀ values, which were computer-generated using a nonlinear regression formula on the GraphPAD program (Institute for Scientific Information), were converted to K_i values using a K_D value for [³H]PIA of 1.0 nM (Jacobson *et al.* 1987) and the Cheng-Prusoff equation (Cheng *et al.* 1973).

Affinity at rat striatal A_{2A} receptors was measured by two methods, using either [³H]NECA or [³H]CGS21680. Inhibition of binding of 5'-N-[³H]ethylcarboxamidoadenosine (specific activity 18 Ci/mmol) to A_{2A} adenosine receptors in rat striatal membranes was measured as described (Bruns *et al.*, 1986), except that 5 mM theophylline was used to define non-specific binding. N⁶-cyclopentyladenosine was present at 50 nM to inhibit binding of the ligand at A₁ adenosine receptors. IC₅₀ values were converted to K_i values as described (Bruns *et al.*, 1986). Use of [³H]CGS21680 (specific activity 48.1 Ci/mmol) as an A_{2A} radioligand, precluded the need for adding N⁶-cyclopentyladenosine (Hutchison *et al.* 1989). Rat striatum was homogenized in 25 volumes of ice cold 50 mM Tris adjusted to pH 7.4 with hydrochloric acid containing 10 mM magnesium chloride, using a polytron (Kinematica, GmbH., Luzerne, Switzerland) at a setting of 2–3 for 10s. The membrane suspension was then centrifuged at 37 000 × g for 20 min at 4° C. The pellet was resuspended in buffer containing 2 IU/mL adenosine deaminase Type VI from calf intestinal mucosa (Sigma, St. Louis, MO, USA) to 20 mg/mL original tissue weight, and incubated at 37°C for 30min. The membrane homogenate was recentrifuged as before, and the pellet was stored frozen at –70°C until use.

For competitive binding assays using [³H]CGS21680, a volume of 1 mL was used in each 13 × 100 mm glass tube. The unlabelled competing ligand of 2-chloroadenosine, for determination of nonspecific binding, was dissolved in 25 pL of DMSO. Then 50pL of 200 m~MgCl₂, 725pL of 50mM Tris (pH7.4 at room temperature) and 100pL of radioligand were added to produce a final concentration of 5 nM. Finally 100 pL of a striatal tissue suspension (final concentration of 150–200 µg protein/mL) was added. The mixture was incubated with shaking for 90 min at 24° C. The samples were filtered on a Brandel Cell Harvester (Brandel, Gaithersburg, MD, USA) with Whatman GF/B filters, and washed rapidly twice with 5 mL of ice cold 50 mM Tris, pH 7.4. Each filter disc was added to 4mL of scintillation fluid, vortexed, and counted after 6 h.

Competitive binding assay in bovine brain using [¹²⁵I]PAPAAPEC—Bovine striatal membranes were prepared as described in Barrington *et al.* (1989). 150 µL of striatal membranes (approximately 0.5 mg protein/mL, suspended in 50 m ~ HEPES buffer at pH 7.2, containing 10 mM MgCl₂) was combined with 50 µL of the indicated competitor and 50 µL of [¹²⁵I]PAPA-APEC to yield a final concentration of radioligand of 1 nM. After a 1 h incubation at 37° C, the mixture was filtered rapidly over No. 32 Schleicher and Scheull glass fiber filters, which had been pretreated for 1 h with 0.3% polyethyleneimine. The

filters were washed with three $\times$ 3 mL aliquots of buffer (pH 7.2), containing 50 mM HEPES, 10 mM MgCl₂, and 0.05% CHAPS. The filters were placed in polypropylene tubes and counted in a Packard gamma counter.

aminoethylaminocarbonylethylphenylethylamino-5'-Nethylcarboxamidoadenosine), was prepared as a common synthetic intermediate for molecular probes of A_{2A} adenosine receptors. The amine was synthesized (Scheme 1 and Table 1) through aminolysis by ethylene diamine of the methyl ester derivative of CGS21680, compound **2**. The amine congener, **3**, was readily acylated, with various activated carboxylic active esters containing prosthetic groups, or with aryl isothiocyanates. The new derivatives were then assayed for activity in binding studies (Table 2) and in a functional assay (Fig. 2).

The small quantities synthesized and the high molecular weights, necessitated the use of high field NMR and californium plasma desorption mass spectroscopy (Jacobson *et al.*, 1986) for characterization of the homogeneous products. The mass spectra (Table 1) for positive ions show either the $[M + H]^+$ or $[M + Na]^+$ ions, or peaks resulting from the loss of the 5'-N-ethylcarboxamidoribose moiety. Thiourea derivatives, obtained from reaction of APEC with isothiocyanates and model compounds for comparison, tended to lose sulfur during measurement of mass spectra. As was observed previously, some of the mass spectra showed the sodium replacement ion of the sodium salt, $[M + 2Na - H]^+$.

Radioactive probes: Towards the goal of radioiodination of functionalized congeners, both phenolic prosthetic groups (Jacobson *et al.*, 1985), such as p-hydroxyphenylpropionic acid (Bolton-Hunter group), and aryl amine-containing prosthetic groups (Stiles *et al.*, 1987), such as p-aminophenylacetic acid (PAPA) have been utilized. An additional prosthetic group for iodination, a 2-substituted thienyl group, which does not contain a hydroxyl or amino group, has been shown to iodinate readily (and selectively in the presence of phenols) via its easily formed mercury adduct (Jacobson *et al.*, 1989b). Thus, the p-hydroxyphenylpropionyl-, **4**, 2-thienylacetyl-, **5**, and p-aminophenylacetyl-, **6**, derivatives of APEC were synthesized as radioiodination substrates (Scheme 1). Compounds **4** and **5** were synthesized via the direct acylation of APEC. Compound **6** was synthesized via the condensation of the

RESULTS

In the development of ligands for molecular characterization of the A_{2A} receptor protein, we adopted a functionalized congener approach, utilizing the carboxyl group of CGS21680 (Compound **1**, Table 1) for attachment of a chemically reactive chain designed not to preclude receptor binding of the derivative. A conceptually similar series of functionalized congeners has been developed for the characterization of A₁ adenosine receptors by radioactive and spectroscopic methods (Jacobson *et al.*, 1987). A carboxyl or amino group at a newly created distal site on an adenosine (agonist) or xanthine (antagonist) derivative, was covalently linked to a reporter group, for probing the receptor binding interaction. Similarly, in the present series of adenosine derivatives, a pivotal amine analog, compound **3** (APEC, carboxylic acid CGS21680 with the appropriate amine, i.e., compound **18**). The resulting benzyloxycarbonylprotected intermediate, **7**, was deprotected through hydrogenolysis. An additional aryl amine, **8**, was prepared via the corresponding N-carbobenzyloxy protected intermediate **9**.

Radiiodinated aryl amine derivatives have an advantage over radiiodinated phenols as molecular probes, in that they may be crosslinked to the receptor protein. Aryl amines, following iodination with **I**, have been photoaffinity crosslinked to A₁ adenosine receptors (Stiles *et al.*, 1985) or converted to azido derivatives which were photolyzed in the receptor

bound state (Stiles et al., 1986; Linden et al., 1988). Compounds **6** and **8** are designed for both radioiodination and photoaffinity crosslinking. Compound **8** was designed in an attempt to overcome low yield in the iodination step (see below), due to possible oxidation at a benzylic methylene group of **6**. Compound **8** is a benzoic acid derivative in which one potentially susceptible α -methylene group is absent. The meta aniline derivative was selected over the para, because of its greater nucleophilicity (required for the crosslinking reaction) based on electronic effects and pK, data in 3-aminobenzoyl derivatives vs the corresponding 4-aminobenzoyl derivatives.

PAPA-APEC, **6**, was radioiodinated by the chloramine T method in 70% radiochemical yield. The product, [^{125}I]PAPA-APEC, having a specific activity of approximately 2200 Ci/mmol, was purified by reverse phase high pressure liquid chromatography (Fig. 1). The two main radioactive peaks were identified using TLC as [^{125}I]PAPA-APEC (peak B) and recovered [^{125}I]iodide. The major ultraviolet absorbing peak (A) corresponded to unreacted compound **6**.

[^{125}I]PAPA-APEC displayed a 60–80% degree of specific binding to striatal $\text{A}_{2\text{A}}$ adenosine receptors in the bovine brain with a K_d value of 1.5 nM (Barrington *et al.*, 1989). [^{125}I]PAPA-APEC was used in photoaffinity crosslinking followed by SDS gel electrophoresis, determining the molecular weight of this receptor to be 45 000 (Barrington *et al.*, 1989). Here we further demonstrated the utility of this A_2 selective iodinated radioligand in competitive binding assays. Typical binding curves for displacement of [^{125}I]PAPA-APEC for bovine brain membranes are shown in Fig. 3.

A prosthetic group for radiofluorination of functionalized drugs and peptides was recently reported (Shai *et al.*, 1989). This group consisted of a 4-fluoromethylbenzoyl moiety (FMB) obtained through nucleophilic fluorination (with ^{18}F) of the corresponding 4-bromomethylbenzoyl derivatives. As a potential “F probe for *in vivo* positron emission tomography (PET) of $\text{A}_{2\text{A}}$ adenosine receptors, an FMB derivative of CGS21680, compound **16**, in which the spacer chain consists of 1,4-diaminobutane, was prepared. It was synthesized by condensing the 4-(4-fluoromethyl)benzoylamino)-butaneamino (Shai *et al.*, 1989) with CGS21680.

Non-radioactive probes

APEC, **3**, was acylated with other reported groups suitable for non-radioactive methods of receptor characterization. Biotin/avidin technology has been used to isolate receptors by affinity chromatography on immobilized avidin columns (Finn *et al.*, 1988) and for histochemistry. A biotin conjugate of APEC, **10**, was synthesized for this purpose.

The amine-functionalized congeners XAC and ADAC, which are antagonist and agonist probes, respectively, for A_1 receptors, have been converted to irreversibly binding ligands for the receptor (Stiles *et al.*, 1988; Jacobson *et al.*, 1989a) through chemical activation using hetero- and homo-bifunctional crosslinking reagents. Examples of bifunctional reagents used successfully in that capacity are the *p*- and *m*- isomers of phenylenediisothiocyanate (DITC). Here, we synthesized *m*- and *p*-DITC-APEC, compounds **11** and **12**, respectively, as potentially chemically reactive affinity labels. A bromoacetyl N^6 -substituted derivative of adenosine was found to inhibit A_1 adenosine receptors irreversibly (Jacobson *et al.*, 1989a). Here we have prepared a bromoacetyl derivative, **14**, designed to inhibit $\text{A}_{2\text{A}}$ adenosine receptors.

A charged thiourea derivative, **13**, containing a sulfonate salt, was prepared. This conjugate, which is approximately as potent and selective as CGS21680, is expected to be prevented

from crossing the blood/brain barrier, due to its completely charged state at physiological pH.

Spin label and fluorescent analogs of purine functionalized congeners, having K_i values at A_1 adenosine receptors in rat brain ranging from 4 to 40 nM, have been synthesized as A_1 receptor probes (Jacobson *et al.*, 1987). A spin label probe, compound **15**, containing the stable free radical TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy), for detection using electron spin resonance spectroscopy (Blanton *et al.*, 1988), was prepared in an analogous fashion from APEC, and the isothiocyanate derivative of TEMPO.

Assays of receptor affinity and adenylate cyclase

The adenosine analogs were assayed for affinity at A_1 adenosine receptors using the radioligands N^6 [^3H]-phenylisopropyladenosine and N^6 -[^3H]cyclohexyladenosine. At A_{2A} receptors, three methods were used for comparison: 1, inhibition of binding of 5'- N -[^3H]ethylcarboxamidoadenosine to rat striatal membranes, according to the method of Bruns *et al.* (1986), in which the A_1 -selective ligand N^6 -cyclopentyladenosine is added to eliminate the A_1 receptor component of specific binding; 2, inhibition of binding of [^3H]CGS21680 to rat striatal membranes, and 3, inhibition of binding of [^{125}I]-PAPA-APEC to bovine brain striatal membranes. Striatal membranes from the calf were used for the following reasons: 1, bovine striatum has a higher density of A_1 adenosine receptors than does the rat (1.1 vs 0.5 pmol receptors per mg of protein); 2, larger quantities of striatum are more readily dissected from calf brains than from rat brains; 3, bovine A_{2A} receptors are more stable to storage and have more favorable levels of nonspecific binding of radioligands.

At A_{2A} adenosine receptors, the derivatives retained high affinity, comparable to the agonist CGS21680, from which they were derived. CGS21680 had a K_i value of 14 nM in inhibition of binding of [^{125}I]PAPA-APEC. Compounds **2,3,7,9, 11**, and **12** displayed K_i values less than 10 nM in displacement of binding of [^{125}I]PAPAAPEC from bovine brain A_{2A} adenosine receptors. Compounds **6, 8**, and **16** were less potent in displacing [^{125}I]PAPA-APEC binding. The affinity of the aminefunctionalized congener, compound **3** (APEC), was unaffected at A_{2A} receptors but was enhanced substantially at A_1 receptors. At A_{2A} adenosine receptors, the amine congener APEC, **3**, is 26-fold more potent than CGS21680, **1**, a carboxylic acid derivative. At A_{2A} adenosine receptors, APEC is similar in potency to CGS21680. Thus, in binding assays the amine congener APEC is not as A_{2A} -selective as CGS21680 or the immediate precursor, the methyl ester of CGS21680, **2**. A distal benzyloxycarbonyl group was well tolerated at A_{2A} receptors, as evidenced from the affinity of compounds **7** and **9**, in comparison to the corresponding free aryl amines, compounds **6** and **8**. The aryl amine derivatives both displayed moderate affinity (K_i values approximately 40 nM) at A_1 receptors, which would likely be enhanced upon iodination. An iodo substituent on the aniline ring probably enhances potency, in view of the K_d value of 1.4 nM observed for [^{125}I]PAPA-APEC (derived from compound **6**). Among the iodlatable analogs synthesized, the *p*-hydroxyphenylpropionyl (Bolton-Hunter) derivative, **4**, had the highest affinity at A_1 receptors (equal to the affinity of CGS21680). Thus, the reaction of APEC with the Bolton-Hunter reagent (commercially available in the radioiodinated form) is a viable route to another iodinated radioligand.

The aryl isothiocyanates (DITC conjugates), **11** and **12**, and the bromoacetyl derivative, **14**, are potential affinity labels for A_{2A} receptors. Both isothiocyanate isomers displayed relatively high affinity at A_{2A} receptors, with apparent K_i values nearly equal to the K_i value for the synthetic precursor, APEC. The *meta* isomer, **11**, was particularly potent in [^3H]NECA binding.

The biotin conjugate, **10**, and a p-sulfonate derivative, **13**, displayed the highest A_{2A} selectivity (at least 350-fold) of the compounds prepared. The precise selectivity value of **10** was not determined, since at A₁ receptors, the highest concentration used in the binding assay (M) caused less than 50% inhibition of specific binding of the radioligand.

A comparison of *K_i* values derived from displacement of binding of [¹²⁵I] PAPA-APEC and [³H]NECA, show some difference (eg., for NECA and compounds **2** and **11**), but no clear trend is apparent. In spite of the species difference (Stone *et al.*, 1988), there is a fair correspondence in *K_i* values using the two methods. The differences in *K_i* values are generally less than threefold, except for NECA and R-PIA, which appear to be fivefold and sevenfold less potent, respectively using I-PAPA-APEC in the bovine brain. At A₁ receptors (Ukena *et al.*, 1986) a large species dependence has been observed for certain N⁶-substituted adenosine analogs as well, and also for many C-8 aryl xanthine derivatives.

Although the high affinity at A_{2A} receptors is maintained in general as the functionalized chain is lengthened, the high A_{2A} selectivity observed for CGS21680 and the methyl ester, **2**, is often diminished. This occurs mainly as a result of increased affinity at A₁ receptors rather than loss of affinity at A_{2A} receptors. In general, the A_{2A} affinity of the conjugates synthesized was relatively constant, with *K_i* values for [¹²⁵I]PAPA-APEC binding in the range of 6 to 43 nM. Conversely, the affinity at A₁ receptors was subject to greater variation, with *K_i* values ranging from 70 to > 5000 nM. Thus, the A_{2A} adenosine receptor is less sensitive to structural changes at this distal site than is the A₁ adenosine receptor. Biological activity of the adenosine agonists in a functional assay was also examined. The ability of compound **6** to increase cyclic AMP in human platelets (Newman *et al.*, 1978) is shown in Fig. 2. A dose-dependent increase in cyclic AMP was observed (Fig. 2) over the range of concentration of compound **6** of 1 nM to 10 μM. At 10⁻⁶ M compound **6** produced a 40% increase in the level of cyclic AMP. At the same concentration, NECA produced a comparable 35% rise in cyclic AMP.

DISCUSSION

We have shown that CGS21680 may be derivatized as an amine derivative (APEC) through the inclusion of an aminoethylamide group. This amine derivative retains high affinity for A₁ receptors, albeit with diminished selectivity. APEC has been subsequently coupled to a variety of reporter groups, resulting in high affinity probes for detection and characterization of A₁ adenosine receptors. The new conjugates of high molecular weight were active in receptor binding assays and in a functional assay as A₁ adenosine agonists (stimulation of cyclic AMP in platelets). These probes include analogs for radiolabelling, a biotin conjugate, ligands for chemical affinity labelling (bearing electrophilic groups), photoaffinity crosslinking (aryl amines), and a free radical derivative for electron spin resonance spectroscopy. This series includes chemically reactive chains potentially of use in anchoring the high affinity ligands to a solid matrix, for isolation of A₁ receptors by affinity chromatography. One aryl amine derivative was iodinated using ¹²⁵I, to afford an A_{2A}-selective agonist radioligand of high affinity and high specific activity.

As with previous findings with ligands selective for A₁ receptors (Jacobson *et al.*, 1985), the presence of a bulky functionalized chain on adenosine analogs need not interfere with a high affinity interaction at A_{2A} receptors. This chain, here located at the C-2 position of adenosine 5'-carboxamides (based on CGS21680) may be extended, in effect creating new distal sites for derivatization. The desirable pharmacological potency in this series of derivatives is not eliminated upon structural extension. Moreover, the conjugates reported here are potentially useful as high affinity molecular probes for receptor characterization by

radioactive, spectroscopic, or affinity labelling methodology, and for receptor isolation by affinity chromatography.

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Abbreviations used

CGS21680	2-(4-(2-carboxyethyl)phenylethylamino)-5'-N-ethylcarboxamidoadenosine
NECA	5'-N-ethylcarboxamidoadenosine
PIA	N ⁶ -phenylisopropyladenosine
E DAC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
CHAPS	3-((3-cholamidopropyl)dimethylammonio)- I -propane sulfonate

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Figure 1.
HPLC elution profile for the synthesis of [^{125}I]PAPA-APEC. Peaks A and B correspond to compound **6**, and [^{125}I]PAPA-APEC, respectively.

Figure 2.

Dose-dependent increase in cyclic AMP in human platelets produced by a potent A_2 adenosine agonist, compound **6**. This curve is typical of three experiments, each being a triplicate assay. Basal cyclic AMP production was $28.3 \text{ pmol mg}^{-1} \text{ min}^{-1}$. At $1 \text{ } \mu\text{M}$, NECA gave a response of $38.1 \text{ pmol mg}^{-1} \text{ min}^{-1}$ representing a 35% increase in cyclic AMP.

Figure 3. Competition curves for the displacement of $[^{125}\text{I}]\text{PAPA-APEC}$ from A_2 adenosine receptors in bovine brain membranes. The concentration of $[^{125}\text{I}]\text{PAPA-APEC}$ was 1 nM. IC_{50} values were as follows: NECA, 51 nM; R-PIA, 1290 nM; compound 6, 9.1 nM; compound 10, 28.9 nM; compound 16, 62.2 nM.

Scheme 1.

Synthetic routes to derivatives of CGS21680 as adenosine receptor probes.

Table 1

Structures of 2,5'-disubstituted adenosine derivatives synthesized, and their characterization by californium plasma desorption mass spectroscopy.

R	%Yield)	MS Peaks
1 OH (CGS21680)	-	544[M + Na ₂ -H] ⁺ , 522, #393, ^c 371 ^c
2 -OCH ₃	-	536, ^a 514, ^b 341, ^c 336, 163
3 -NH(CH ₂) ₂ NH ₂ (APEC)	91	564, ^a 542, ^b 369, ^c 336, 163
4 -NH(CH ₂) ₂ NHCO(CH ₂) ₂ -	82	712, ^a 690, ^b 539, ^c 517, ^c 336, 163
5 -NH(CH ₂) ₂ NHCOCH ₂ -	96	689, ^a 667, ^b 163
6 -NH(CH ₂) ₂ NHCOCH ₂ -	68	697, ^a 546, ^c 524, ^c 163
7 -NH(CH ₂) ₂ NHCOCH ₂ -	65	831, ^a 809, ^b 658, ^c 336
8 -NH(CH ₂) ₂ NHCO-	87	697 ^b
9 -NH(CH ₂) ₂ NHCO-	70	817, ^a 795, ^b 336, 163
10 -NH(CH ₂) ₂ NHCO(CH ₂) ₄ -	82	791, ^a 769 ^b
11 -NH(CH ₂) ₂ NHCSNH-	47	756, ^a 734, ^b 724 [M + Na - S], 702 [M + H ⁺ - S]
12 -NH(CH ₂) ₂ NHCSNH-	44	734, ^b 702
13 -NH(CH ₂) ₂ NHCSNH-	85	802, ^a 764 ^c
14 -NH(CH ₂) ₂ NHCOCH ₂ Br	57	685 ^a
15 -NH(CH ₂) ₂ NHCSNH-	69	724 [M + H - S], 564, 550, ^c 336, 163
16 -NH(CH ₂) ₂ NHCO-	81	728, ^a 706, ^b 555, ^c 534, ^c 336, 163, 130

^a [M + Na]⁺

^b [M + H]⁺

^c Loss of 173(5'-N-ethylcarboxamidoribose) from [M + Na]⁺, [M + H⁺ - S], or from [M + Na₂-H]⁺

Table 2

Potencies of adenosine derivatives and xanthines at adenosine A₁ and at A₂ adenosine receptors in binding assays^a

Compound	A ₁ receptors		A ₂ receptors			$\frac{K_i(A_1)}{K_i(A_2)}$
	(³ H)PIA rat	[¹²⁵ I] PAPA APEC bovine	[³ H]NECA rat	[³ H]CGS21680 rat		
NECA	6.26 ± 0.52 ^b	55 ± 26	10.3 ± 0.5 ^b	12 ± 1		0.11
R-PIA	1.17 ± 0.16 ^b	870 ± 270	124 ± 9 ^b	410 ± 32		0.0013
S-PIA	49.3 ± 2.4 ^b	10 300 ± 2800	1820 ± 380 ^b	3 020 ± 210		0.0048
ADAC	0.85	(Nd) ^d	210	218 ± 28		-
Theophylline	8470 ± 1490 ^b	20 300 ± 2700	25 300 ± 200 ^b	20 800 ± 1540		0.417
XAC	1.2 ± 0.5	(Nd) ^d	44	30.7 ± 1.7		-
1	2600 ± 300 ^c	14.1 ± 2.1	15	14 ± 1		184
2	2260 ± 440 ^c	6.1 ± 1.5	17.5 ± 1.6	5 ± 2		290
3	99.4 ± 11.6	6.1 ± 1.3	5.73 ± 0.52	12 ± 3		16
4	950 ± 100 ^c	13.2 ± 1.5	(Nd) ^d	13 ± 4		72
5	1400 ± 100 ^c	21.3 ± 7.9	(Nd) ^d	15 ± 1		66
6	1340	43 ± 14	(Nd) ^d	28 ± 7		31
7	278 ± 18.7	6.2 ± 0.9	5.59 ± 0.99	37 ± 5		45
8	1340 ± 28	43.6 ± 5.2	(Nd) ^d	16.5 ± 2.8		31
9	680 ± 110 ^c	8.7 ± 2.0	(Nd) ^d	12 ± 2		78
10	> 5000 ^c	14.3 ± 3.3	(Nd) ^d	55 ± 30		>350
11	69.7 ± 7.9	6.2 ± 2.1	2.82 ± 0.38	22 ± 7		11
12	276 ± 75 ^c	7.1 ± 2.3	(Nd) ^d	35 ± 10		39
13	3780 ± 800	10.9 ± 2.3	(Nd) ^d	26 ± 10		347
14	1980 ± 102	15.8 ± 4.8	(Nd) ^d	69.7		125
15	177 ± 38 ^c	14.8 ± 3.6	(Nd) ^d	27 ± 3		13
16	1450 ± 67	25.4 ± 7.6	(Nd) ^d	18.9 ± 4.9		57

^aUnless noted, expressed as the K_1 value in nM, for inhibition of binding of [³H] PIA at A₁ receptors, inhibition of binding of [¹²⁵I] PAPA-APEC, [³H]NECA, or [³H]CGS21680 at A₂ receptors. Compounds **11**, **12**, and **14** are potential irreversible inhibitors of A₂ receptors, thus the values given represent apparent K_1 's. Values are the means ± SEM for three or more determinations in triplicate. The A₂ selectivity ratio derived from K_1 values for [¹²⁵I] PAPA-APEC binding at A₂ receptors.

^bData from Bruns *et al.* (1986); inhibition of binding of N⁶-[³H] cyclohexyladenosine at A₁ receptors, inhibition of binding of [³H]NECA at A₂ receptors.

^cUsing N⁶-[³H] cyclohexyladenosine.

^dNot determined

^eThe ratio of K_1 values at A₁ receptors vs the K_1 values in the [¹²⁵I] PAPA-APEC assay.