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MOLECULAR PROBES FOR EXTRACELLULAR ADENOSINE RECEPTORS

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Abstract

Derivatives of adenosine receptor agonists (N^6-phenyladenosines) and antagonists (1,3-dialkyl-8-phenylxanthines) bearing functionalized chains suitable for attachment to other molecules have been reported [Jacobson *et al.*, *J. med. Chem.* **28**, 1334 and 1341 (1985)]. The "functionalized congener" approach has been extended to the synthesis of spectroscopic and other probes for adenosine receptors that retain high affinity ($K_i \sim 10^{-9} - 10^{-8}$ M) in A_1-receptor binding. The probes have been synthesized from an antagonist xanthine amine congener (XAC) and an adenosine amine congener (ADAC). [^{3}H]ADAC has been synthesized and found to bind highly specifically to A_1-adenosine receptors of rat and calf cerebral cortical membranes with K_D values of 1.4 and 0.34 nM respectively. The higher affinity in the bovine brain, seen also with many of the probes derived from ADAC and XAC, is associated with phenyl substituents. The spectroscopic probes contain a reporter group attached at a distal site of the functionalized chain. These bifunctional ligands may contain a spin label (e.g. the nitroxyl radical TEMPO) for electron spin resonance spectroscopy, or a fluorescent dye, including fluorescein and 4-nitrobenz-2-oxa-1,3-diazole (NBD), or labels for ^{19}F nuclear magnetic resonance spectroscopy. Potential applications of the spectroscopic probes in characterization of adenosine receptors are discussed.

"Functionalized congener" refers to a drug derivative that contains a chemical functional group, such as an amine or carboxylic acid, attached through a spacer chain to a position on the pharmacophore, which position is relatively insensitive to steric bulk. The congeners are designed as intermediates for the synthesis of covalent conjugates that retain binding properties at the receptor site for the drug. The aims of this approach are 2-fold: (1) to develop potential new pharmaceutical agents in which the activity of the primary pharmacophore (portion of drug molecule essential for biological activity) may be modulated through distal changes, and (2) to synthesize radioactive and non-radioactive molecular probes for the drug binding site. Since the strategy of this drug design allows groups to be placed at a distance from the binding site of the primary pharmacophore, it is possible to overcome constraints on the size of the attached group [1–5].

Sites for functionalization of adenosine receptor ligands have been identified for both agonists (derivatives of N^6-phenyladenosine) and antagonists (derivatives of 1,3-dialkyl-8-phenylxanthine). A 1,3-dipropylxanthine amine congener, **1** (XAC[‡]), has been tritiated [4] and found to have K_D values for binding to A_1-adenosine receptors in bovine and rat brain

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of 0.17 and 1.2 nM respectively. Thus, [^{3}H]XAC is the first generally useful antagonist radioligand for adenosine receptors. Moreover, XAC can be attached to carrier molecules [3], such as amino acids and peptides, to form covalent conjugates having high receptor affinity.

Similarly, we have developed an adenosine amine congener, **2** (ADAC), having a K_i of binding against [^{3}H]N^6-cyclohexyladenosine in rat cerebral cortex membranes of 0.85 nM [2]. A common structure–activity relationship (SAR) feature in both agonist and antagonist series is that the presence of a distal amino group located on the attached chain in many cases results in increased affinity to adenosine receptors.

In this study we have examined the parameters of binding of tritiated ADAC and have demonstrated the utility of both ADAC and XAC as synthetic precursors for receptor probes. Biotin-containing probes for adenosine receptors have been reported [6]. Based on the impressive nanomolar affinity of ADAC for adenosine A_1-receptors and a very low level of nonspecific binding, this in spite of the presence of the two lipophilic phenyl groups in the chain, we have synthesized ADAC conjugates as probes for studying molecular properties and localization of adenosine receptors. These probes contain markers for fluorometric, spin-label, nuclear magnetic resonance, and other spectroscopic methods of detection. The range of potent derivatives of XAC has been expanded to include similar conjugates as antagonist-derived probes.

EXPERIMENTAL PROCEDURES

Materials

N_6-*R*-Phenylisopropyladenosine (*R*-PIA) and 5′-*N*-ethylcarboxamido adenosine (NECA) were purchased from Research Biochemicals Inc., Natick, MA. [^{3}H]N^6-*R*-Phenylisopropyladenosine (49.9 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Theophylline, adenine and inosine were from the Sigma Chemical Co., St. Louis, MO. 2′,5′-Dideoxyadenosine was from P-L Biochemicals, Milwaukee, WI, and dipyridamole was from Thomae, Biberach, FRG. All other chemicals were obtained from sources described previously [1, 2, 4].

The NMR spectra (measured on a Varian 300 MHz spectrophotometer) of new compounds synthesized were consistent with the assigned structures. Fluorescence spectra were measured on a SPEX Fluorolog 2 spectrometer.

Preparation of [^{3}H]ADAC (2)

Unlabeled ADAC [2], 7 mg, was dissolved in 0.1 M sodium phosphate, pH 10, and subjected to catalytic exchange [9] using 100 mg of 5% PdO/$BaSO_4$ under 10 Ci of tritium gas (carried out by the Amersham Corp., U.K., procedure code TR.7). The catalyst was removed by filtration, and labile protons were exchanged, leaving 33 mCi of radioactivity, 25% of which co-migrated with ADAC by thin-layer chromatography (Merck silica gel 60, $CHCl_3$–MeOH–HOAc, 10:10:1, silica, R_f = 0.14). Most of the radioactive impurities were less polar than ADAC. [^{3}H]ADAC (retention time 9.7 min) was purified by HPLC using an Altex Ultra-sphere ODS 5 μm column (0.46 × 25 cm) with a mobile phase of 50% methanol in 10 mM triethylammonium trifluoroacetate (1.0 ml/min). The recovery for the purification step was 15%. Thus, the overall yield of isolated [^{3}H]ADAC (purity 96%) was only 0.4%.

‡Abbreviations: ADAC, adenosine amine congener, **2**; CHA, N^6-cyclohexyladenosine; DTPA, diethylenetriaminepentaacetic acid; FITC, fluorescein isothiocyanate; NBD, 4-nitrobenz-2-oxa-1,3-diazole; NECA, 5′-*N*-ethyl-carboxamidoadenosine; *R*-PIA, *R*-phenylisopropyladenosine; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy radical; TRITC, tetramethylrhodamine isothiocyanate; and XAC, xanthine amine congener, **1**.

The concentration was determined by u.v. spectroscopy using an ε-value of 32,400 for the absorption peak at 303 nm. The specific activity was calculated to be 27.5 Ci/mmol.

The crude product from the tritium exchange reaction could be purified efficiently also by ion exchange chromatography. After dilution with an equal volume of water, the mixture was passed over a cation exchange column (2 ml) in the hydrogen form (Amberlite IRC-50), and washed with 5 ml water. Elution with 2 N ammonium hydroxide (10 ml) gave pure ADAC (20% of radioactivity applied to column). With unlabeled ADAC, this procedure resulted in 95% recovery.

Synthesis of receptor probes

Fluorescein-containing probes, **3–5**, were synthesized by acylation of a dimethylformamide (DMF) suspension (10–20 mg/ml) of the appropriate amine derivative (e.g. 8 - [4 - [[[[(2-aminoethyl)amino]carbonyl]methyl]-oxy]phenyl]-1,3-dipropylxanthine, **1**; N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]anilino]-carbonyl]methyl]phenyl]adenosine, **2**; or **18**) with a 30–50% molar excess of a pure isomer of fluorescein isothiocyanate (see Ref. 10, Aldrich Chemical Co., Milwaukee, WI). The reactions were monitored by thin-layer chromatography. When the free amine congener was no longer detectable, the solvent was evaporated under a stream of argon, and the product was isolated and purified by two crystallizations from DMF/methanol/ether.

Probes containing other covalently-bound fluorescent dyes, e.g. **6** and **7**, were prepared similarly from 7-chloro-4-nitrobenz-2-oxa-1,3-diazole chloride (NBD Chloride, Sigma, see Ref. 11), or from tetramethylrhodamine isothiocyanate, isomer *R* (Sigma) (see Ref. 12), respectively. Californium plasma desorption mass spectra [5] for compounds **6** and **7** showed intense positive ion peaks at 762 and 1042 mass units, respectively, corresponding to $(M + Na)^+$ ions, and smaller $(M + H)^+$ peaks at 741 and 1020 m.u.

Metal complexing probes, **12** and **13**, were prepared similarly using diethylenetriaminepentaacetic (DTPA) anhydride (Sigma), introduced by Hnatowich *et al.* [13]. The Californium mass spectrum for compound **13** showed major peaks at 827 $(M + Na)^+$ and at 805 $(M + H)^+$ m.u.

TEMPO-ADAC (8)

ADAC, **2** (11.8 mg, 20 μmol), was suspended in 0.5 ml DMF and treated with 4-isothiocyanato-TEMPO (2,2,6,6,-tetramethyl-1-piperidinyloxy, free radical, 7 mg, 33 μmol, Aldrich). After 1 hr, 1.5 ml water was added to the solution, and the precipitate was collected, washed with a minimum of MeOH and ether, and recrystallized from DMF/ether/petroleum ether, to give a product (8.3 mg, 51%) which was homogeneous by thin-layer chromatography and gave proton NMR and ESR spectra consistent with the structure. Californium plasma desorption MS peak [5] at 758 (M + 1 − MeOH) and 626 (M + 1 − ribose). The IR spectrum showed a peak at 1580 cm^{-1}, characteristic of a thiourea carbonyl stretch.

N-Trifluoroacetyl-XAC (10)

XAC, **1** (27 mg, 64 μmol), was dissolved in a mixture of DMF (10 ml) and diisopropylethylamine (1 ml) and treated with 1ml of ethyl trifluoroacetate. The reaction was warmed (50°) for 10 min. Upon addition of 10 ml of H_2O, the product precipitated, giving 21 mg (64% yield) of *N*-trifluoroacetyl-XAC, NMR in $(CD_3)_2SO$ δ 9.46 (1H, CF_3CONH), 8.31 (1H, NH), 8.08 (d, 2H, J = 8.8 Hz, Ar), 7.09 (d, 2H, J = 8.8 Hz, Ar), 4.55 (s, 2H, CH_2O), 3.29 (m, 4H, CH_2CH_2NH). Analysis ($C_{23}H_{27}N_6O_5F_3$): calc. 52.67% C, 5.19% H, 16.02% N; found 52.48% C, 5.23% H, 15.97% N.

N-Heptafluorobutyryl-XAC (11)

Heptafluorobutyryl anhydride (0.5 ml, Aldrich) was added to a mixture of DMF (5 ml), EtOH (2 ml), and diisopropylethylamine (1 ml). XAC (29 mg, 68 μmol) was added, and the mixture was heated (50°) overnight. Water was added, and the mixture was extracted three times with ethyl acetate. The organic layer was washed with sodium bicarbonate and pH 6 phosphate buffer and evaporated leaving 29 mg of *N*-heptafluorobutyryl-XAC. Recrystallization from ethyl acetate/petroleum ether gave 17 mg. The proton NMR spectrum was consistent with the assigned structure. Analysis ($C_{25}H_{27}N_6O_5F_7$): calc. 48.08% C, 4.36% H, 13.46% N; found 49.33% C, 4.88% H, 13.78% N.

Benzyloxycarbonyl-triglycyl-XAC (17)

Cbz-glycyl-glycyl-glycine (90 mg, 0.28 mmol, Sigma), XAC, **1** (42 mg, 0.10 mmol), and 1-hydroxybenzotriazole (30 mg, 0.22 mmol) were combined in 2 ml of DMF and treated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (80 mg, 0.37 mmol, Sigma). After the mixture was stirred for 24 hr, addition of 4 ml of H_20 caused the product, compound **17**, to precipitate (73 mg, 100%), m.p. 229–233°. The product was homogeneous by thin-layer chromatography. Analysis ($C_{35}H_{41}N_9O_9{\cdot}H_2O$): calc. 56.07% C, 5.78% H, 16.81% N; found 55.78% C, 5.84% H, 16.73% N. NMR in $(CD_3)_2SO$ δ8.23 (t, 1H, NH), 8.16(m, 2H, NH), 8.08(d, 2H, J = 8.8Hz, Ar), 7.88 (t, 1H, NH), 7.51 (t, 1H, NH), 7.35 (s, 5H, phenyl), 7.10 (d, 2H, J = 8.8 Hz, Ar), 5.02 (s, 2H, CH_2Ar), 4.55 (s, 2H, CH_2OAr), 3.75 (d, 2H, J 5.5 Hz, CH_2Gly), 3.76 (d, 4H, CH_2Gly), 3.18 (m, 4H, CH_2CH_2NH).

Compound **17** was deprotected with 30% HBr/acetic acid for 1 hr at room temperature to give triglycyl-XAC hydrobromide, **18**, in nearly quantitative yield, m.p. 250–255 dec. Analysis ($C_{27}H_{36}N_9O_7Br{\cdot}4H_2O$): calc. 43.20% C, 5.91% H, 16.79% N; found 43.37% C, 5.55% H, 16.94% N.

N^{α}-9-Fluorenylmethyloxycarbonyl-N^{ε}-benzyloxycarbonyl-D-lysine (20)

ε-Benzyloxycarbonyl-D-lysine was acetylated with 9-fluorenemethyloxycarbonyl chloride in dioxane/aqueous sodium carbonate according to the method of Carpino and Han [14] to give compound **20** in 90% yield as a clear glass, which solidified upon heating at 60% in a vacuum oven, m.p. 83–87°. Analysis ($C_{29}H_{30}N_2O_6{\cdot}½H_2O$): calc. 68.09% C, 6.11% H, 5.48% N; found 68.05% C, 6.15% H, 5.07% N.

N^{ε}-9-Fluorenylmethyloxycarbonyl-D-lysine (21)

Compound **20** (0.70 g, 1.4 mmol) was treated with 30% HBr/acetic acid for 1 hr. Upon evaporation, the resulting oil was purified by preparative thin-layer chromatography ($CHCl_3$/MeOH/HOAc, 70:25:5) to give compound **21** as an amorphous glass (0.14g) in 27%yield.

N^{α}-9-Fluorenylmethyloxycarbonyl-N^{ε}-biotinyl-D-lysine (22)

A mixture of FMOC-D-lysine, **21** (83 mg, 0.23 mmol), *N*-succinimidyl biotin (88 mg, 0.23 mmol), and diisopropylethylamine (39μl, 0.23 mmol) in 5 ml of dimethylformamide/tetrahydrofuran (2:1 mixture) was stirred overnight. Ethyl acetate and 0.1 M HCl were added, and the phases were mixed and separated. The upper phase was washed (H_2O) and evaporated, and the residue was triturated with ethyl acetate and petroleum ether. The solid product (63 mg, 47%), melting at 139–143°, was collected. Chemical ionization mass spectrometry (CIMS) and NMR were consistent with the assigned structure. Analysis ($C_{31}H_{38}N_4O_6S{\cdot}5/2H_2O{\cdot}½DMF$): calc. 57.72% C, 6.93% H, 9.32% N; found 57.39% C, 6.28% H, 9.86% N.

N^{ε}-Biotinyl-D-lysyl-ADAC (14)

Compound **20** (28 mg, 47 μ mol) was treated with 1-hydroxybenzotriazole (6mg, 47 μmol) and dicyclohexylcarbodiimide (10 mg, 48 μmol) in DMF (ice cold). ADAC, **2** (17.8 mg, 31 μmol), was added, and the reaction was stirred for 10 hr. The mixture was chromatographed on a small column of LH-20, eluting with methanol. Fractions which contained the product, compound **23** (identified as biotin-containing spot on TLC, see Ref. 6), were pooled and treated with 2 vol. of ether and 1 vol. of petroleum ether. A homogeneous white solid (5 mg) was collected and identified by proton NMR. Compound **23** was deprotected by treatment with a 10% solution of diethyl-amine in dimethylformamide to give compound **14**. The NMR spectrum was consistent with the assigned structure.

Purification

Some of the molecular probes could be purified to thin-layer chromatographic homogeneity by successive recrystallizations (see Table 2). Others were purified readily by preparative thin-layer chromatography. Several conjugates containing highly polar or charged attached "carriers", e.g. the derivative of the fluorescent dye tetramethylrhodamine, **7**, required other methods of purification. Thus, compound **3** was further purified by semipreparative high pressure liquid chromatography (Lichrosorb RP-18, 60% MeOH/10mM triethylammonium trifluoroacetate), and compound **7** was purified by the continuous counter current method introduced by Ito [15] using a solvent mixture of chloroform/acetic acid/methanol/DMSO/water in the ratio of 5:1:1:1:3.

Preparation of membranes

Membranes from rat and calf cerebral cortex were prepared according to the method of Whittaker [8] as described previously [4]. Rat pheochromocytoma (PC12) cell and human platelet membranes were prepared as described [7,16]. The protein concentration was determined according to Lowry *et al.* [17].

Radioligand binding

The binding of [^{3}H]ADAC to cerebral cortex membranes from rat and calf was measured in a total volume of 1 ml containing 50 mM Tris-HCl, pH7.4, 0.2 units adenosine deaminase and approximately 50–100 μg of membrane protein. The radioligand was routinely present in a final concentration of 1 nM. Other substances were added as indicated. Incubation was carried out at 37° for 120 min. All assays were done in triplicate. Bound and free radioligand were separated by addition of 4 ml of ice-cold incubation buffer followed by rapid filtration through Schleicher & Schuell GF/B glass fiber filters that had been treated with 0.3% polyethylenimine for 60 min as described by Bruns *et al.* [18]. The filters were washed twice with 5 ml of ice-cold incubation buffer. For filtration, a Brandel M-24R manifold (Brandel Instruments, Gaithersburg, MD) was used. Nonspecific binding of [^{3}H]ADAC was determined in the presence of 10 μM *R*-PIA. At 1 nM [^{3}H]ADAC, nonspecific binding amounted to about 10–15% with rat and about 5% with calf cerebral cortex membranes. The same amount of nonspecific binding was obtained with 5 mM theophylline. Nonspecific binding of 1 nM [^{3}H]ADAC to filters amounted to about 4% of the total counts filtered with untreated filters and was nearly completely eliminated with polyethylenimine-treated filters.

Binding of 1 nM [^{3}H]PIA to rat and calf cerebral cortex membranes was carried out essentially in the same way. Nonspecific binding was less than 10% of total binding. Radioligand binding data were analyzed as described [4]. Slope factors were determined from Hill plots ("pseudo-Hill" coefficients).

Adenylate cyclase assay

Adenylate cyclase was assayed essentially as described [7,16]. Briefly stated, the medium contains 0.1 mM [α-^{32}P]ATP (0.3 μCi/tube), 1μM GTP, 1mM $MgCl_2$, 0.1 mM cyclic AMP, 1 μg/ml adenosine deaminase, 0.1 mM rolipram (ZK 62,711), 1 mM ethyleneglycolbis-(amino-ethylether)tetra-acetate (EGTA), 5 mM creatine phosphate as the Tris-salt, 0.4μg/ml creatine kinase, 2 mg/ml bovine serum albumin and 50 mM Tris–HCl, pH 7.4, in a total volume of 100 μl. Incubations were initiated by the addition of 10–15 μg of membrane protein and were conducted for 10 min at 37°. Cyclic AMP was purified as described [7].

RESULTS

ADAC, **2** had been synthesized as a versatile intermediate for the preparation of a broad series of additional analogs through coupling reactions at the terminal amino group. Prior to coupling of reporter groups of ADAC, we prepared [^{3}H]ADAC to investigate the binding parameters of this functionalized congener. This ligand is comparable to other widely used commercial N^6-substituted-adenosine radioligands (e.g. *R*-PIA) in that it combines high affinity with a low degree of nonspecific binding, in spite of added lipophilic groups on the N^6-substituent.

The tritiated form of an adenosine amine congener, ADAC (Fig. 1), was synthesized by the catalytic exchange method of Evans *et al.* [9]. The method calls for the stirring of a nucleotide or saccharide derivative in an aqueous medium (basic pH) in the presence of a large mass excess of the hydrogenation catalyst, 5% palladium oxide supported on barium sulfate. In our case the chemical yield of the reaction, estimated to be 15–20% before purification by HPLC, was lower than expected for this tritiation method. This low yield may result from adsorption of the product to the catalyst, since there is no indication that the product, [^{3}H]ADAC, is particularly labile in aqueous buffer systems of moderate pH (7–8). Based on the work of Evans *et al.* [9], five protons of ADAC should be most susceptible to tritium exchange—the C-8 proton of adenine and the four benzylic protons. However, the observed specific activity of 27.5Ci/mmol suggests substitution mainly at the most readily exchanged C-8 proton.

Saturable binding to rat and calf cerebral cortex membranes of [^{3}H]ADAC of high specific activity was readily demonstrated (Fig. 2). In both tissues nonspecific binding increased linearly with radioligand concentrations. Specific [^{3}H]ADAC binding was saturable with B_{max} values of 0.57 and 0.64 pmol/mg protein in rat and calf cerebral cortex respectively. Nearly identical B_{max} values have been obtained using the agonist ligand [^{3}H]PIA and the antagonist ligand [^{3}H]XAC [4]. Scatchard analysis revealed binding of [^{3}H]ADAC to single binding sites. [^{3}H]ADAC bound to rat cerebral cortex membranes with a K_D of 1.4 nM. The binding affinity in calf cerebral cortex (K_D of 0.34 nM) was about 4-fold higher.

Competition experiments with adenosine agonists and antagonists show that [^{3}H]ADAC binding occurred to A_1 receptors. All of the competition curves were monophasic, indicating an interaction at a single binding site (Fig. 3). The agonists CHA, ADAC and *R*-PIA and the antagonist XAC were the most potent inhibitors of the binding of [^{3}H]ADAC to rat cerebral membranes (Table 1). The K_i values of these compounds for inhibition of [^{3}H]ADAC binding are in excellent agreement with the K_D values for these compounds determined in saturation experiments (see Fig. 2, and Refs. 4 and 19). *R*-PIA was a more potent inhibitor of binding of [^{3}H]ADAC than NECA, a characteristic typical of brain and fat cell A_1 receptors.

XAC was about 9-fold more potent than its parent compound, 1,3-dipropyl-8-phenylxanthine, in competing for [^{3}H]ADAC binding sites. The K_i values of the antagonists

are nearly identical to those obtained for inhibition of [^{3}H]CHA or [^{3}H]XAC binding to the same membrane preparation [4,19].

For inhibition of [^{3}H]ADAC binding to calf cerebral cortex membranes, an order of potency identical to that found in the rat was obtained with the series of ligands (Table 1), but the absolute values differed. Previous studies have shown that 8-phenyl-xanthine derivatives have higher affinities to A_1-adenosine receptors of calf brain compared to rat brain [4,19]. Competition experiments with [^{3}H]ADAC confirmed these observations (Table 1). Incorporation of *P*-sulfo substituents reduced the potency of 8-phenylxanthine derivatives more in calf than in rat brain. In both tissues, compounds with no intrinsic activity at adenosine receptors inhibited [^{3}H]ADAC binding only slightly, even at very high concentrations (Table 1).

The biological activity of ADAC at A_2 adenosine receptors was assessed in adenylate cyclase studies in membranes from cells containing A_2 receptors [7,16]. In rat pheochromocytoma (PC12) cells, ADAC stimulated adenylate cyclase activity with an EC_{50} of 800 nM, a value 8-fold higher than that of NECA (Fig. 4A). In these membranes, ADAC was fully as efficacious as NECA. In human platelet membranes, ADAC was about 4-fold less potent and also less efficacious than NECA (Fig. 4B). These differences in efficacy of adenosine analogs for stimulation of platelet adenylate cyclase also have been observed for other adenosine derivatives; few analogs are as efficacious as NECA in stimulating cyclase activity in these membranes [20]. The activities of ADAC and its derivatives as A_2 agonists in cardiac vasodilation also have been reported [21]. In comparison to the EC_{50} values for adenylate cyclase stimulation, the binding characteristics of [^{3}H]ADAC show that this functionalized congener of adenosine was about 600- to 700-fold selective for A_1 receptors.

Thus, ADAC is an ideally suited agonist for the design of bifunctional probes containing reporter groups because of its high potency at adenosine receptors and the distal location of the primary amino group. Previous studies [1–3,6] indicated that a wide variety of prosthetic groups could be attached covalently to the amino group of ADAC or XAC without preventing receptor binding. A series of molecular probes was synthesized (Table 2) from ADAC and XAC, as agonist and antagonist ligands, respectively, for extracellular adenosine receptors, based on the general strategy of attachment of functionalized congeners to reporter groups. The molecular probes were purified as described in Experimental Procedures and characterized spectroscopically by TLC (Table 3).

Among the probes included in this study (Fig. 5) are fluorescent probes, **3–7**, spin label probes, **8** and **9**, probes for ^{19}F nuclear magnetic resonance spectroscopy, **10** and **11**, and derivatives of the metal chelator, diethylenetriaminepentaacetic acid (DPTA), **12** and **13.** Compounds **3**, **4**, and **6–13** were synthesized in single step reactions involving acylation or arylation (compound **6** only) of the primary amino group of XAC or ADAC. Compounds **5**, **14**, **15**, and **23** were synthesized by the routes shown in Fig. 6. Potentially irreversible probes based on chemical or photochemically reactive prosthetic groups also have been synthesized from XAC and ADAC [5].

Competitive binding assays using [^{3}H]ADAC as a radioligand showed that high affinity at the A_1-adenosine receptor was maintained in most of the molecular probes (Table 4, probes **3**, **6**, **8–11,** and **14** of particularly high potency).

The fluorescence emission spectra for ADAC derivatives **3**, **6**, and **7** in aqueous medium showed maxima at wavelengths characteristic for conjugates of fluorescein isothiocyanate (517 nm), NBD (540 nm), and tetramethylrhodamine isothiocyanate (574 nm). Excitation maxima (494, 470, and 557 nm, respectively) were also characteristic for these structures.

The concentration of compound **3** could be measured in solutions (pH 7.2) as dilute as 10^{-11} M.

The ESR spectra of compounds **8** and **9** at 10^{-6} M in DMSO show three intense peaks, characteristic of piperidinyl radicals [22]. Using a Varian EPR spectrometer E-109 (time const. 0.5 sec, gain 1.25×10^5, power 10 mW, 4min scan, sample in a 50 μl capillary), a signal/noise ratio of 10 was observed.

^{19}Fluorine NMR spectra for XAC derivatives **10** and **11** were measured in $(CD_3)_2SO$. Resonances at 88.19 ppm from hexafluorobenzene for the tri-fluoromethyl group **10** (singlet) and at 82.41, 42.91, and 33.00 ppm for the heptafluoropropyl group of **11** (multiplets) appeared.

The two biotin-containing probes were designed to improve upon a previous study ([6], and see below). After preincubation with a saturating quantity of avidin (1.0 μM), the IC_{50} of compound **15** shifted from 170 to 530 nM. The latter corresponds to a K_i value of 265 nM for the protein complex.

DISCUSSION

A schematic representation of the advantage of the functionalized congener approach for attachment of bulky groups is shown in Fig. 7. Similar groups attached directly to the primary pharmacophore would likely interfere with binding. In addition to the potential interference with binding of a ligand from interactions (steric or otherwise) from distal sites of the attached “carrier”, there is the possibility that an energetically favorable interaction will occur with the receptor molecule, thus stabilizing the bound state. For example, we have demonstrated that a distal amino group located on a chain on various adenosine receptor agonists and antagonists enhances binding [1–3]. We suggest an electrostatic interaction, as depicted in Fig. 7D, as a mechanism for the enhanced affinity.

The higher affinity of N^6-phenyl substituted adenosine analogs, such as ADAC derivatives, and of C^8-phenyl substituted xanthines, such as XAC derivatives, for bovine compared to rat A_1 adenosine receptors was evident (Table 1). Interspecies differences in binding of ligands to A_1-adenosine receptors have been noted [4]. Thus, in studies with the adenosine receptor probes introduced here, bovine tissue sources, instead of rat, may be preferred because of the likely higher affinity of the probe in bovine preparations. The phenyl group appears to be an essential structural requirement for higher potency at bovine receptors, both in xanthines (for a variety of substitutions on purine nitrogens, e.g. 8-phenyl-theophylline and 8-phenyl-1,3-dipropylxanthine) and adenosine analogs (even when the phenyl group is separated from the 6-amino group, e.g. N^6-*R*-phenylisopropyladenosine). Thus N^6-cyclohexyl-adenosine, theophylline, and caffeine are equipotent in bovine and rat brain membranes [4].

Given the tolerance of A_1 receptors for bulky groups, as previously demonstrated for the adenosine receptor with amino acids and peptides in a series of 1,3-dipropylxanthine derivatives [3], and for biotin–avidin complexes in the series of adenosine derivatives [6], the synthesis of other molecular probes, including spectral probes, in both agonist and antagonist series was carried out. For certain types of studies, an antagonist probe may be preferred because of the single, high affinity class of antagonist binding sites. For other studies, in which conformation changes occurring between states of the receptor exhibiting high and low affinity agonist binding sites are examined, agonist probes may be preferred.

Fluorescent analogs of receptor ligands have a variety of applications [23,24], For example, spectral changes of fluorescent analogs have been used to characterize receptor properties in conformations associated with different functional states [12], accessibility of sites by

fluorescence quenching, fluorescence depolarization, and energy transfer, and the distribution and lateral mobility of receptors in membranes using fluorescence photobleaching of NBD derivatives [11]. The direct visualization of receptors for thyrotropin-releasing hormone on a tumor cell line using fluorescent drug analogs has been reported [10]. However, histochemical studies [25] using fluorescent ligands for adrenergic and opiate receptors revealed that the distribution of fluorescence does not reflect the labeling of receptor, but instead the occurrence of lipofuscin, an endogenous fluorescent compound.

We have identified fluorescein and NBD conjugates of ADAC, **3** and **6**, respectively, as high affinity fluorescent ligands for A_1-adenosine receptors. The tetramethylrhodamine conjugate, **7**, was only moderately potent at rat brain A_1 receptors. In the antagonist series, a pair of fluorescent conjugates of XAC, **4** and **5**, differing only in the length of the spacer chain, were compared. In rat brain, the longer chain analog had a slightly higher affinity. These analogs are good candidates for a variety of studies as described above, including fluorescent cell sorting to select populations of cells with a high density of receptors, and examining accessibility using quenching of the fluorescent dye moiety, perhaps with antibodies to fluorescein as reported [26].

ESR has been used widely to characterize binding to and conformational states of proteins and cell components [22,27,28]. A variety of spin-labeled analogs of drugs have been synthesized and characterized [27]. Nitroxide-bearing analogs of cholinergic agonists have been used to describe the kinetics of agonist-mediated transitions of membrane-bound nicotinic receptors [28]. The agonist derivative TEMPO-ADAC, **8**, and the antagonist derivative TEMPO-XAC, **9**, have high affinity at A_1 adenosine receptors. The 4-substituted TEMPO group was selected due to the absence of chiral centers, the presence of which would lead to diastereoisomers in adenosine conjugates. Given a sufficient quantity of receptor (approx. 10^{-6}M required at present), compounds **8** and **9** are potentially useful in ESR studies.

Drug molecules containing fluorine have been used to probe interactions with macromolecules using ^{19}F NMR [22,29]. Other fluorine-containing agonist and antagonist adenosine receptor ligands have been reported [1,2]. Here we have shown that fluorine can be introduced through a prosthetic labeling group coupled to a functionalized congener, e.g. the antagonist XAC, to give compounds **10** and **11**. Due to the relatively low sensitivity of this instrumental method, to carry out ^{19}F NMR with adenosine receptors would require purification of the receptor. However, in this regard, it should be noted that ^{19}F NMR studies have already been reported for the 4-trifluoro-2,6-dinitrophenyl group fixed to whole cells [30].

Prosthetic groups, designed for facile introduction of a particular radioisotope, notably the *p*-hydroxy-phenylpropionyl group for iodination, have been coupled to functionalized congeners with retention of high potenty [1,16]. The strong chelator, DTPA [13,31], is used to complex a variety of heavy metal radioisotopes, such as indium and technetium, to proteins, including monoclonal antibodies, for diagnostic and therapeutic purposes. We attempted to use the DTPA prosthetic group to make available heavy metal analogs of adenosine receptor agonists, **12**, and antagonists **13**. However the affinities of these multiple Zwitterionic conjugates were less than desirable in a receptor probe.

Several biotin conjugates of ADAC with spacer groups of varying length were found to bind simultaneously to the A_1-adenosine receptor and to avidin, a glycoprotein of molecular weight 66,000 [6]. The K_i value for one of the macromolecular conjugates against [^{3}H]CHA binding to rat cerebral cortex membranes was 36 nM. The two biotin-containing probes

were designed to improve upon a previous study [6]. In compound **14**, a free amino group was included on the chain in an attempt to take advantage of interaction with the presumed distal anionic location near the receptor binding site. The potency of **14** in A_1 receptor binding in rat brain (K_i=8.9 ± 5.1 nM) was, in fact, greater than that of the analog in which the amino group is absent (K_i = 18 ± 1.7 nM, biotin-ε-aminocaproyl-ADAC in Ref. 6). The XAC conjugate, **15**, represents a further effort to obtain an antagonist conjugate capable of binding simultaneously to the A_1-adenosine receptor and to avidin. Previous xanthine conjugates (chain length up to 21 Å) could not bind simultaneously to both the receptor antagonist site and avidin, whereas adenosine conjugates with even shorter spacer chains could bind simultaneously to receptor agonist sites and avidin. With a chain length in the fully extended conformation estimated to be 32 Å, compound **15** appears to be capable of binding simultaneously to the receptor and to avidin, although the diminished affinity will limit its utility.

The use of ADAC and XAC as intermediates in the synthesis of a wide range of bifunctional molecular probes for adenosine receptors, some of exceedingly high affinity, has been demonstrated. This study may also serve as a model for the design and synthesis of molecular probes for other receptors using a functionalized congener approach.

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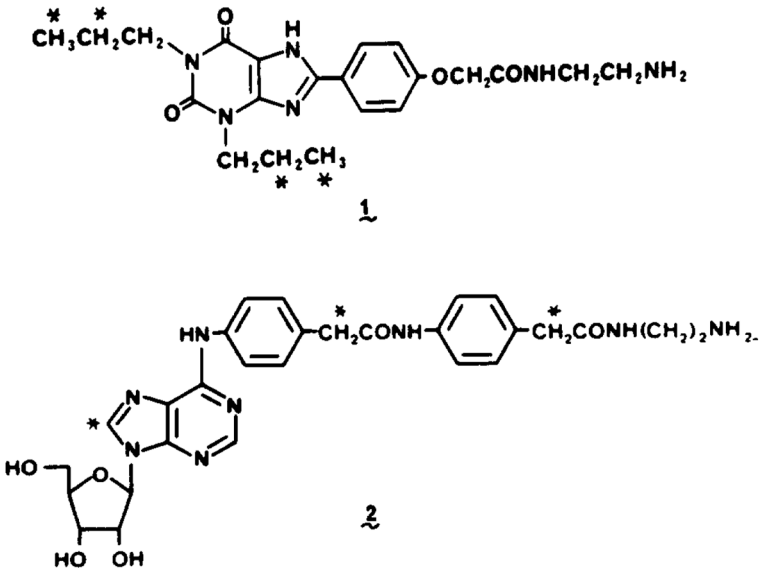

Fig. 1.

Structures and sites of tritiation of the functionalized congeners which are potent adenosine receptor antagonist (**1**, XAC) and agonist (**2**, ADAC) analogs.

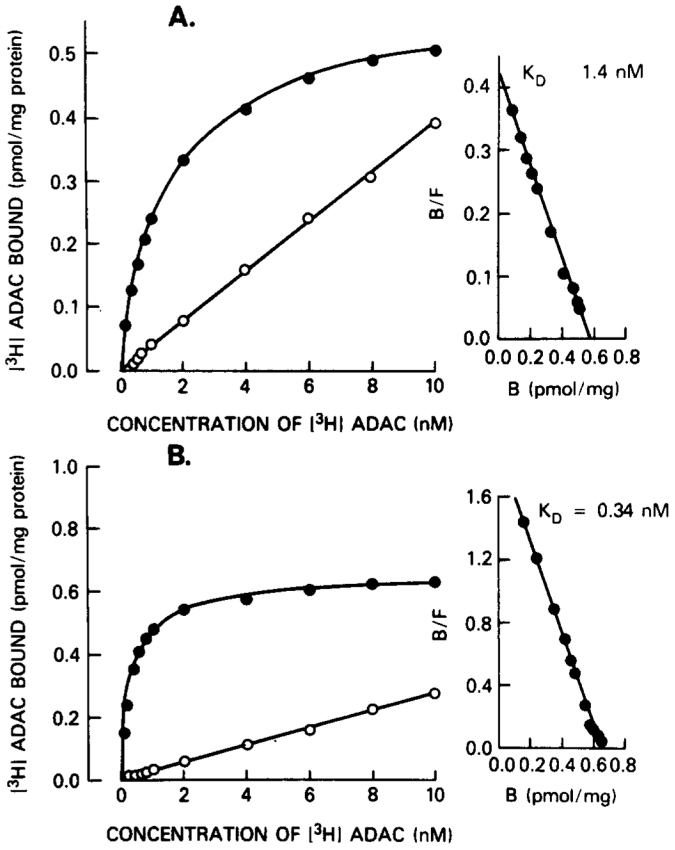

Fig. 2.

Saturation of [^{3}H]ADAC binding to cerebral cortex membranes from rat (A) and calf (B). Specific (●— ●) and nonspecific (○— ○) binding was determined for 120 min at 37°. Values are means of a typical experiment done in triplicate. Right panels: Scatchards plots of the same data. K_D (nM) and B_{max} (pmol/mg protein) were 1.4 and 0.57, respectively, for rat and 0.34 and 0.64, respectively, for calf cerebral cortex membranes.

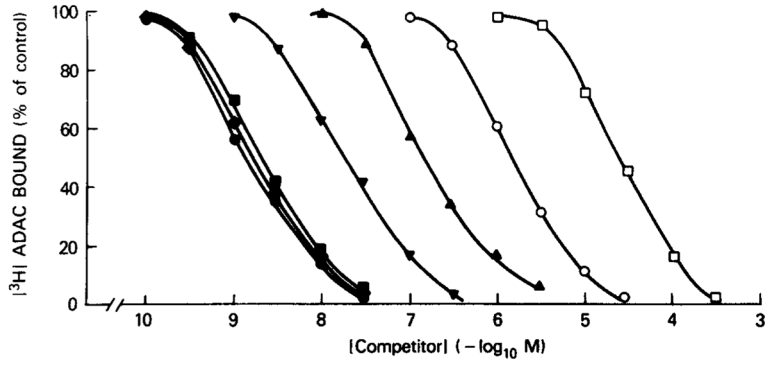

Fig. 3.

Inhibition of [^{3}H]ADAC binding to rat cerebral cortex membranes by adenosine agonists and antagonists. Binding of 1 nM [^{3}H]ADAC was measured for 120 min at 37°. Values are from a typical experiment done in triplicate. Slope factors were 0.98 for ADAC (●); 0.94 for *R*-P1A (◆); 1.01 for XAC (■); 0.98 for NECA (▼); 1.02 for1,3-dipropyl-8-(*p*-sulfophenyl)xanthine (▲); 0.80 for 8-(*p*-sulfophenyl)theophylline (○); and 0.94 for theophylline (□).

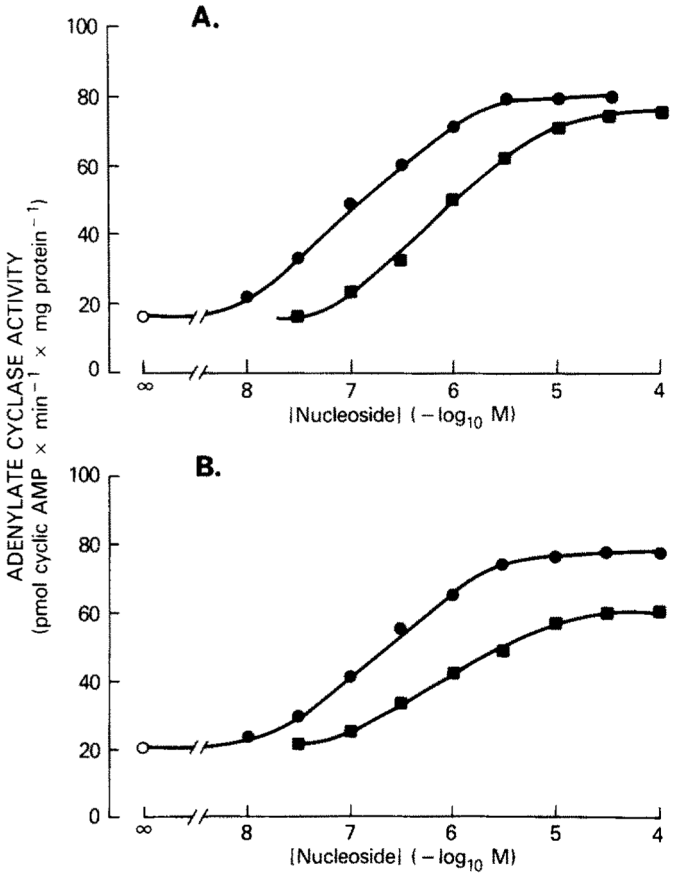

Fig. 4.

Effect of adenosine analogs on adenylate cyclase activity of rat pheochromocytoma (PC12) cell (A) and human platelet (B) membranes. Adenylate cyclase was measured for 10 min at 37°. Values are means of a typical experiment done in triplicate. The EC_{50} values were 100 and 240 nm for NECA (●— ●) and 800 and 980 nM for ADAC (■— ■) in PC12 and platelet membranes, respectively.

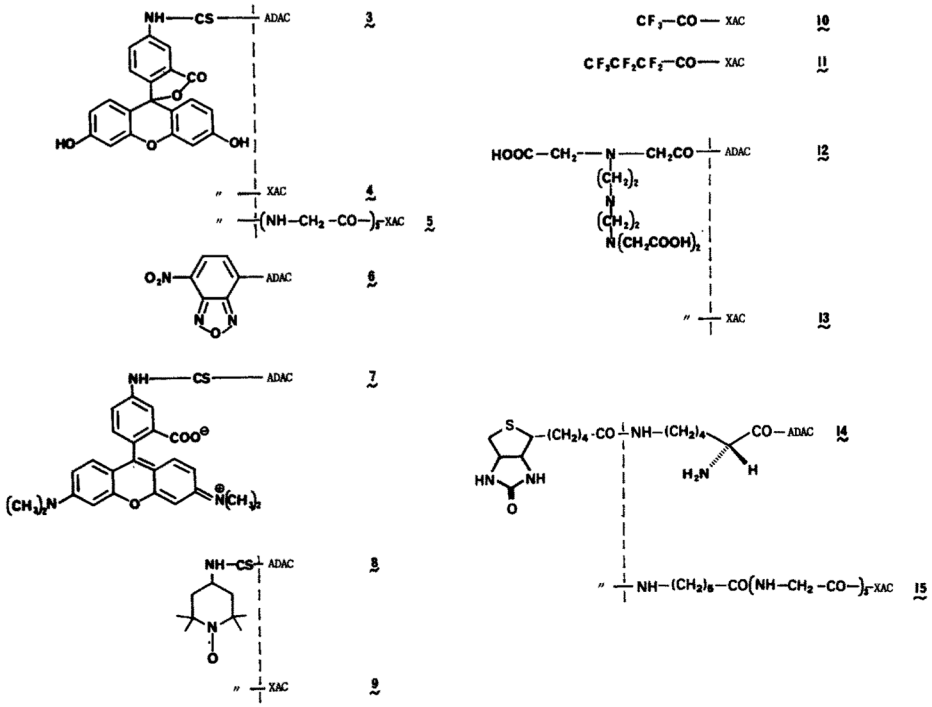

Fig. 5.

Structures of molecular probes for adenosine receptors derived from the functionalized congeners ADAC and XAC (Fig. 1) through coupling at the primary amino group.

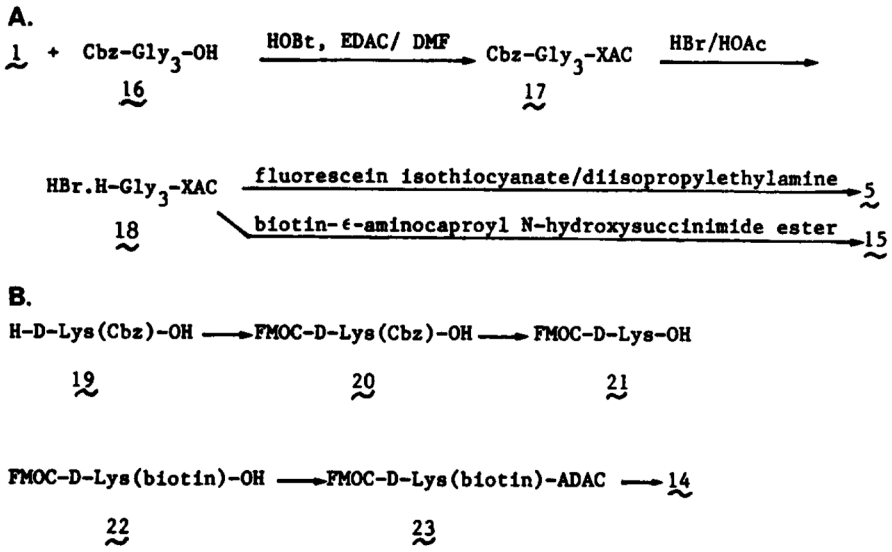

Fig. 6.

Multistep syntheses of several molecular probes containing an extended chain linking the reporter group to an adenosine receptor antagonist (A) or agonist (B).

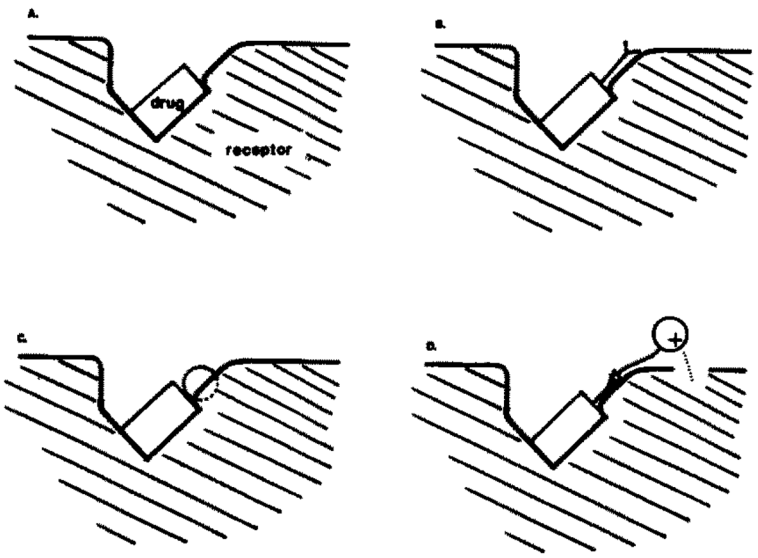

Fig. 7.

Schematic representation of binding of functionalized congeners at a receptor site. (A) A subset of the surface of the drug is exposed to the medium. (B) An analog containing a chain on the exposed portion is synthesized. This “functionalized congener” contains a chemical functional group ( ), such as an amine or carboxylic acid, for the covalent attachment to carrier molecules. (C) The presence of a bulky group close to the pharmacophore may prevent binding. (D) Attachment of a bulky group through the functionalized chain. The attached moiety (carrier) may enhance affinity due to an energetically favorable interaction, either specific or nonspecific, at a distal (accessory) site. At adenosine receptors the presence of a positively-charged ammonium group is associated with high affinity, possibly reflecting a distal electrostatic interaction.

Table 1

Competition for [^{3}H]ADAC binding to rat and calf cerebral cortex membranes

	K_i (nM)	
Compound	**Rat**	**Calf**
CHA	1.2 (0.65–2.4)	1.15 (0.45–2.9)
ADAC	1.3 (0.92–1.90)	0.46 (0.19–1.1)
R-PIA	1.3 (0.84–2.1)	0.78 (0.30–2.0)
NECA	8.6 (4.5–16.5)	50 (45–54)
XAC	1.4 (0.86–2.3)	0.14 (0.07–0.27)
1,3-Dipropyl-8-phenylxanthine	9.2 (4.3–19.3)	0.17 (0.07–0.27)
8-Phenyltheophylline	66 (45–96)	6.8 (3.0–15.5)
1,3-Dipropyl-8-*p*-sulfophenyl xanthine	100 (82–130)	24.5 (20.6–29.1)
8-*p*-Sulfophenyltheophylline	750 (670–830)	250 (210–290)
Theophylline	12,400 (7,600–20,400)	12,500 (5,300–29,600)
Caffeine	41,000 (28,000–60,000)	41,000 (28,000–86,000)
Dipyridamole	28,700 (26,100–31,500)	> 100,000 (27.8%)
Adenine	> 100,000 (42%)	> 100,000 (6.4%)
2′,5′-Dideoxyadenosine	> 100,000 (40%)	> 100,000 (8.8%)
ATP	> 100,000 (35%)	> 100,000 (10.9%)
Inosine	> 100,000 (20.6%)	> 100,000 (1.5)

Competition curves were measured as described in Experimental Procedures. Data are presented as geometric means with 95% confidence limits, in parentheses, from three to four experiments. For K_i values above 100 μM, the percentage inhibition of [^{3}H]ADAC binding at 100 μM is given in parentheses.

Table 2

Reaction yields and characterization of probes

Compound	Abbreviation	Method*	Recryst. solvent	% Yield	M.p. (°)
3	FTTC-ADAC	a	DMF/Et_2O	73†	213–216
4	FITC-XAC	a	MeOH/Et_2O/pet. ether	60	d begin 228
5	FITC-Gly_3-XAC	a	MeOH/Et_2O	67	191–194 d
6	NBD-ADAC	b	DMF/MeOH/H_2O	20	178–181
7	TRITC-ADAC	a	DMF/MeOH/Et_2O	57‡	
8	TEMPO-ADAC	a	DMF/H_2O	51	d begin 178
9	TEMPO-XAC	a	DMF/Et_2O	40	
10	CF_3CO-XAC	g	DMF/H_2O	64	304–306
11	C_3F_7CO-XAC	g	EtOAc/pet. ether	68§	231–234
12	DTPA-ADAC	c	DMF/MeOH/Et_2O	86	163–169
13	DTPA-XAC	c	MeOH/Et_2O	48	179–182 d
14	H(*ε*-Biotin)-D-Lys-ADAC	d	DMF/Et_2O	71	
15	Biotin-Gly_3-XAC	e	DMF/Et_2O	99	221–225 d
23	FMOC-(*ε*-Biotin)-D-Lys-ADAC	f	MeOH/Et_2O/pet. ether	14†	

*Key: (a) isothiocyanate, (b) aryl chloride, (c) carboxylic anhydride, (d) diethylamine/DMF, (e) *N*-hydroxy-succinimide ester, (f) carbodiimide/1-hydroxybenzotriazole, and (g) ethyl ester.

†Including purification by column chromatography on Sephadex LH-20, eluting with methanol.

‡Further purification by continuous counter current chromatography [15].

§Crude yield.

Table 3

Characterization of molecular probes by thin-layer chromatography on Analtech silica gel GF, 250 μm, unless noted*

Compound	R_f (system)		
1	0.57(A)	0.35(B)	0.86(E)
2	0.14(A)	0.93(E)	
3	0.74(B)	0.06(C)	
4	0.96(A)	0.28(B)	0.96(C)
5	0.82(B)	0.17(C)	
6	0.79(B)	0.14(C)	0.86(E)
7	0.78(A)	0.38(B)	0.53(E)
8	0.86(B)	0.17(C)	
9	0.79(C)	0.55(E)	
10	0.81(C)	0.80(D)	0.63(E)
11	0.83(C)	0.85(D)	0.43(E)
12	0.01(A)	0.88(E)	
13	0.11(B)	0.72(E)	
14	0.40(B)		
15	0.81(A)	0.63(B)	0.61(E)
23	0.81(A)	0.72(B)	

*(A) $CHCl_3$–MeOH–HOAc, 10:10:1; (B) $CHCl_3$–MeOH–HOAc, 70:25:5; (C) $CHCl_3$–MeOH–HOAc, 85:10:5; (D) EtOAc–HOAc, 10:1; and (E) Analtech RPS-F, 40% isopropanol/H_2O.

Table 4

Potency of binding of molecular probes at A_1-adenosine receptors in two species using [^{3}H]*R*-PIA as a radioligand (assay as described in Experimental Procedures)

	K_i (nM)		
Compound	**Rat brain**	**Bovine brain**	**K_i (rat)/k_i (bovine)**
1	1.3 ± 0.08	0.15 ± 0.02	8.7
2	1.3 ± 0.08	0.39 ± 0.03	3.3
3	7.1 ±0.3	2.85 ± 0.15	2.5
4	125 ± 27	9.3 ± 0.10	13.4
5	96.5 ± 33.5	16.7 ± 0.2	5.8
6	4.3 ±0.10	1.6 ± 0.0	2.7
7	41.6 ± 6.5	12.9 ± 2.1	3.2
8	4.4 ± 0.15	1.4 ± 0.2	3.1
9	4.9 ± 1.5	0.30 ± 0.12	16
10	4.6 ± 0.45	0.34 ± 0.04	14
11	8.1 ± 1.2	0.83 ± 0.06	9.8
12	109 ± 6.0	24.2 ± 1.15	4.5
13	59.5 ± 20.5	3.25 ± 0.75	18
14	8.9 ± 5.1	2.1 ± 0.30	4.2
15	49.5 ± 4.5	2.95 ± 0.35	16.9