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“Cleavable Trifunctional” Approach to Receptor Affinity Labeling: Chemical Regeneration of Binding to A₁-Adenosine Receptors

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Abstract

A general approach for reversible affinity labeling of receptors has been developed. The objective is to carry out a series of chemical modifications resulting in a covalently-modified, yet functionally-regenerated, receptor protein that also may contain a reporter group. The ligand recognition site of A₁-adenosine receptors in bovine brain membranes was probed to demonstrate the feasibility of this approach. Use of disulfide or ester linkages, intended for cleavage by exposure of the labeled receptor to either reducing reagents or hydroxylamine, respectively, was considered. Binding of the antagonist radioligand [³H]CPX was preserved following incubation of the native receptor with 3 M hydroxylamine, while binding was inhibited by the reducing reagent dithiothreitol (DTT) with an IC₅₀ of 0.29 M. Hydroxylamine displaced specific agonist ([³H]PIA) binding in a noncovalent manner. Specific affinity labels containing reactive isothiocyanate groups were synthesized from XCC (8-[4-[(carboxymethyl)-oxy]phenyl]-1,3-dipropylxanthine) and shown to bind irreversibly to A₁-receptors. The ligands were structurally similar to previously reported xanthine inhibitors (*e.g.*, DITC-XAC: (1989) *J. Med. Chem.* 32, 1043) except that either a disulfide linkage or an ester linkage was incorporated in the chain between the pharmacophore and the isothiocyanate-substituted ring. These groups were intended for chemical cleavage by thiols or hydroxylamine, respectively. Radioligand binding to A₁-receptors was inhibited by these reactive xanthines in a manner that was not reversed by repeated washing. Hydroxylamine or DTT restored a significant fraction of the binding of [³H]CPX in A₁-receptors inhibited by the appropriate cleavable xanthine isothiocyanate derivative.

INTRODUCTION

Affinity labeling is a widely used technique for the characterization of receptor proteins (1). It is particularly useful for membrane-bound proteins which are not readily characterized by other physical methods, due to the difficulty of isolation in quantities required. By the affinity labeling technique a high affinity ligand that contains a chemically reactive group, such as a bromoacetyl, methylfumaryl, or isothiocyanate (2), or a photochemically reactive group, such as azide (3), is synthesized. The electrophilic group of a chemically-reactive ligand may spontaneously combine with a nucleophilic group that may be present on the binding site of the receptor (4) or on a neighboring molecule in the membrane (5). We have utilized a variety of cross-linking reagents to covalently anchor selective, purine ligands (amine-functionalized congeners) to subtypes of adenosine receptors (2, 6, 7). Generally,

receptor affinity labels have been designed without a detailed knowledge of the environment of the ligand binding site, and the identification of reactive ligands that bind covalently to the receptor protein has been empirical. A radioisotope may be incorporated into the affinity label (6,20) for purposes of receptor detection (*e.g.*, on electrophoretic gels) and imaging.

A deficiency of typical protein labeling is that the modified receptor, although intact in its primary structure, is functionally inactive, both in ligand binding and in activation of second messengers. It would be desirable to have a general means of regenerating the functional binding site following affinity labeling, either in its native form or in a covalently-labeled state. Thus, several groups have introduced reversible affinity labeling schemes (8, 9, 23). Patchornik et al. (8) designed a scheme for the photochemical deblocking of an affinity-labeled biopolymer to regenerate its native form. Denny and Blobel (9) reported an ^{125}I -labeled heterobifunctional protein cross-linking reagent (*N*-[4-[(*p*-azido-*m*-iodophenyl)azo]benzoyl]-3-(aminopropyl)-*N'*-oxysulfosuccinimide ester) that may be cleaved by the action of sodium dithionite on an azo linkage joining the two reactive moieties. The biopolymer is not regenerated in its native form; instead a radioiodine label remains with one component after cleavage. Similarly, disulfide groups are used in a variety of protein and nucleic acid cross-linkers (10), such as biotinylated probes, for subsequent cleavage under reducing conditions.

Adenosine is a neuromodulator that allows organs such as the heart, brain, and kidneys adapt to stress and maintain homeostasis, by acting at specific cell surface G-protein coupled receptors (reviewed in ref 21). As part of a detailed structural investigation of A_1 - and A_{2a} -adenosine receptor subtypes, we introduced a general trifunctional approach to affinity labeling (11). By this approach a reporter group (*e.g.*, radioactive or spectroscopic) is tethered to a receptor ligand, which delivers it to the binding site selectively, and then incorporated covalently onto the receptor protein via a chemically reactive group (an isothiocyanate) present on the same molecule. A key intermediate is a trifunctional cross-linker consisting of a 3,5-diisothiocyanatobenzene derivative (equivalent to structure **2** in Figure 1). One of the isothiocyanate groups reacts with an amine-functionalized ligand, and the other reacts with the receptor protein.

In this study we have expanded this approach conceptually to include chemically-cleavable spacer groups. An amine-functionalized ligand, **1**, may be designed to contain a cleavable A–B linkage (Figure 1). Upon coupling to a trifunctional cross-linker, **2**, a conjugate **3** is obtained. In **3** the cleavage site is located between the trifunctional phenyl ring and the pharmacophore. After receptor binding and cleavage of A–B, a portion of the label remains covalently bound to the receptor protein (structure **4b**). The attached portion contains the reporter group (R) but not the pharmacophore moiety. The pharmacophore may then freely dissociate from the binding site. Thus, the receptor protein remains chemically labeled, and in principle, the receptor binding site is, at least in part, unoccupied and again able to bind radioligands.

EXPERIMENTAL PROCEDURES

Synthesis

^1H NMR spectra were recorded using a Varian XL-300 FT-NMR spectrometer, and all values are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Chemical ionization MS using ionized NH_3 gas were recorded using a Finnigan 1015D mass spectrometer modified with EXTREL electronics. Fast atom bombardment MS was carried out on a JEOL JMS-SX102 mass spectrometer. Thin-layer chromatography (TLC) analyses were carried out using EM Kieselgel 60 F254, DC-Alufolien 200 $\times$ b5 plates and were visualized under ultraviolet light. Elemental analyses were performed by Atlantic Microlabs,

Inc., Atlanta, GA. XCC,¹ *m*-DITC-XAC, and *m*-DITC-ADAC were synthesized as previously reported (2,16). Cystamine dihydrochloride was obtained from Sigma (St. Louis, MO).

N-tert-Butyloxycarbonyl)cystamine, 8—Di-*tert*-butyldicarbonate (0.48 g, 2.2 mmol) and triethylamine (0.91 mL, 3 equiv) were added to a methanolic solution (25 mL) of cystamine bis hydrochloride (0.5 g, 2.2 mmol). After 20 min the solvent was evaporated, and 1 M NaH₂PO₄ was added (10 mL, pH 4.2). The aqueous solution was extracted with ether to remove the di-*t*-Boc-cystamine (mp 106–107 °C, 0.135 g, 17.5%). The aqueous solution was basified to pH 9 by 1 M NaOH and extracted with EtOAc (5 mL × 6). The combined organic phases were dried over MgSO₄ and evaporated to yield the product (0.24 g, 43%). The product was isolated as the hydrochloride salt (mp. 109–110 °C). ¹H NMR (D₂O) δ: 3.40 (q, 4H, CH₂N, *J* = 6.6 Hz), 3.00 (q, 2H, CH₂S, *J* = 7 Hz), 2.87 (t, 2H, CH₂S, *J* = 6.3 Hz), 1.45 (s, 9H, *t*-BuO). MS (CI/NH₃) free amine: 253 (MH⁺) 253 (MH⁺ – *t*-Boc). Anal. Calcd. for C₉H₂₁ClN₂O₂S₂: C, 37.42; H, 7.33; N, 9.70. Found: C, 37.46; H, 7.34; N, 9.61.

8-[4-[[[1-[2-[(*tert*-Butyloxycarbonyl)amino]ethyl]dithio]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine, 10—Mono-*t*-Boc-cystamine (8, 80 mg, 0.32 mmol), EDAC (0.165 g, 0.85 mmol), and 1-hydroxybenzotriazole (0.1 g, 0.75 mmol) were added to XCC (9, 0.125 g, 0.32 mmol) in dry DMF (10 mL). The reaction mixture was sonicated at room temperature for 30 min. EtOAc (10 mL) was added, and the turbid solution was extracted with water and then with 2 M Na₂CO₃ (pH 11). The combined aqueous solutions were extracted with EtOAc (10 mL × 2), dried, and evaporated under high vacuum. The solid obtained was washed with a small amount of ether and dried under high vacuum to yield a light yellowish solid (0.147 g, 75%), mp 184 °C. ¹H NMR (CDCl₃) δ: 8.23 (d, 2H, ArH, *J* = 8.8 Hz), 7.05 (d, 2H, ArH, *J* = 8.8 Hz), 4.95 (br s, 1H, NH), 4.60 (s, 2H, CH₂O), 4.19, 4.11 (t, 2H, CH₂N), 3.72, 3.43 (2H, CH₂NH), 2.90, 2.82 (t, 2H, CH₂S), 1.88, 1.77 (q, 2H, CH₂CH₂), 1.45 (s, 9H, *t*-Boc), 1.02, 0.99 (t, 3H, CH₂CH₃). MS (FAB): 621 (M⁺). Anal. Calcd. for C₂₈H₄₀N₆O₆S₂: C, 54.18, H, 6.49; N, 13.54. Found: C, 54.28; H, 6.54; N, 13.47.

8-[4-[[[1-[(2-Aminoethyl)dithio]ethyl]amino]carbonyl]-methyl]oxy]phenyl]-1,3-dipropylxanthine Trifluoroacetate, 11—Compound 10 (0.047 g, 0.756 mmol) was dissolved in trifluoroacetic acid (1.5 mL), and the solution was stirred at room temperature under nitrogen. After 10 min, the flow rate of nitrogen was increased, and trifluoroacetic acid was evaporated. Ether (5 mL) was added to the glassy residue to form a white solid, which was dried under high vacuum for 48 h (0.044 g, 92%), mp > 230 °C. ¹H NMR (CD₃OD) δ: 8.02 (d, 2H, ArH, *J* = 8.7 Hz), 7.13 (d, 2H, ArH, *J* = 8.7 Hz), 4.83 (s, 2H, OCH₂CO), 4.13, 3.97 (t, 2H, CH₂N, *J* = 7 Hz), 3.63 (t, 2H, CH₂NH₂), 3.50 (q, 2H, CH₂NH, *J* = 7 Hz), 2.98, 2.91 (t, 2H, CH₂S), 1.75, 1.64 (q, 2H, CH₂CH₂), 0.99, 0.95 (t, 3H, CH₂CH₃). Anal. Calcd. for C₂₅H₃₃N₆O₆S₂F₃ (hydro-trifluoroacetate of 11)-1/2H₂O: C, 46.65; H, 5.32; N, 13.06. Found: C, 46.67; H, 5.26; N, 12.77.

8-[4-[[[1-[2-[[[3-Isothiocyanatophenyl]amino]thiocarbonyl]amino]ethyl]dithio]ethyl]amino]car

¹Abbreviations: ADAC, N⁶-[4-[[[4-[[[2-aminoethyl]amino]-carbonyl]methyl]anilino]carbonyl]methyl]phenyl]adenosine; Boc, *tert*-butyloxycarbonyl; Cbz, benzyloxycarbonyl; CPX, 8-cyclopentyl-1,3-dipropylxanthine; DITC, phenylene diisothiocyanate; DTT, dithiothreitol; EDAC, *N*-ethyl-*N'*-(3-diaminopropyl)carbodiimide hydrochloride; EtOAc, ethyl acetate; IBMX, 3-isobutyl-1-methylxanthine; PIA, N⁶-phenylisopropyladenosine; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; XAC, 8-[4-[[[2-aminoethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine; XCC, 8-[4-[[[carboxymethyl]oxy]phenyl]-1,3-dipropylxanthine.

bonyl]methyl]-oxy]phenyl]-1,3-dipropylxanthine, 12—A dry DMF solution (5 mL) of product **11** (0.032 g, 0.053 mmol), triethylamine (0.1 mL, 2 equiv), and *m*-phenylene diisothiocyanate (ref 6, 30 mg, 3 equiv) was stirred at room temperature for 0.5 h. The solvent was removed under high vacuum (bath temperature 36 °C) to leave a semisolid residue, which was purified on a micro silica column (EtOAc + 1% Et₃N), followed by treatment of the product with dry ether and drying under high vacuum (30 mg, 84%), dec 185 °C. The product was homogeneous by TLC (*R*_f = 0.82, silica, using chloroform:methanol:acetic acid 85:10:5, by vol) and reacted with ethylenediamine to form cleanly a new compound of *R*_f 0.30 in the same system. ¹H NMR (CDCl₃) δ: 8.93 (s, 1H, ArH-2), 8.16 (d, 2H, ArH, *J* = 8.7 Hz), 7.34 (m, 1H, ArH), 7.04 (d, 2H, ArH, *J* = 8.7 Hz), 6.96 ('t', 2H, ArH, *J* = 5.2 Hz), 4.60 (s, 2H, CH₂O), 4.17 (t, 2H, CH₂N), 3.73 (q, 2H, CH₂NH), 3.14, 3.12 (q, 2H, CH₂N), 3.02, 2.87 (t, 2H, CH₂S), 1.86, 1.71 (q, 2H, CH₂CH₂), 1.01, 0.96 (t, 3H, CH₂CH₃). Anal. Calcd. for C₃₁H₃₆N₈O₄S₄: C, 52.23; H, 5.09; N, 15.72. Found: C, 51.80; H, 5.26; N, 15.13.

2-[(Benzyloxycarbonyl)amino]ethanol, 13—2-Aminoethanol (0.91 g, 15 mmol) was dissolved in ethyl acetate (30 mL), and sodium carbonate (3 g) was added. The mixture was treated with a solution of benzyl chloroformate (3.3 g, 19 mmol) dissolved in 30 mL of ethyl acetate, added in aliquots with stirring. The mixture was filtered, and the filtrate was treated with water and ethyl acetate. After separation of the layers, the organic layer was extracted with pH 7 phosphate buffer and then with 0.1 M HCl. The organic layer was evaporated to dryness leaving a residue which was triturated with petroleum ether. The resulting solid was collected and recrystallized from ethyl acetate/petroleum ether to provide the product as white crystals (mp 58–59 °C, 36% yield). MS: 214, 196 (*M* + 1). The NMR spectrum was consistent with the assigned structure.

(tert-Butyloxycarbonyl)-β-alanine 2-[(Benzyloxycarbonyl)amino]ethyl Ester, 15—A solution of (*tert*-butyloxycarbonyl)-β-alanine (**14**, 0.98 g, 5.2 mmol) and compound **13** (0.81 g, 4.15 mmol) in DMF (40 mL) was treated with EDAC (1.3 g, 6.8 mmol) and DMAP (0.8 g, 6.6 mmol) with stirring. After 2 h, half-saturated sodium chloride was added. The product was extracted into ethyl acetate, which was washed with 0.1 M HCl and then 0.1 M Na₂CO₃, dried (Na₂SO₄), and evaporated, leaving an oil (0.96 g, 63% yield). MS: 384, 367 (*M* + 1), 328, 311, 267. The NMR spectrum was consistent with the assigned structure.

β-Alanine 2-[(Benzyloxycarbonyl)amino]ethyl Ester Trifluoroacetate, 16—Compound **15** (0.51 g, 1.4 mmol) was dissolved in a minimum volume of trifluoroacetic acid, and the solution was stirred at room temperature under nitrogen. After 30 min, the trifluoroacetic acid was evaporated, and the glassy residue was dried under high vacuum (0.33 g, 63% yield). MS: 267 (*M* + 1), 240, 212. The NMR spectrum was consistent with the assigned structure.

8-[4-[[[1-[[2-[(Benzyloxycarbonyl)amino]ethyl]oxy]carbonyl]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine, 17—Compound **16** (0.15 g, 0.39 mmol), EDAC (0.4 g, 1.8 mmol), and 1-hydroxybenzotriazole (0.05 g, 0.37 mmol) were added to XCC (**9**, 0.12 g, 0.31 mmol) in dry DMF (20 mL). The reaction mixture was sonicated at room temperature for 30 min. Water was added, and a precipitate formed. The solid obtained was washed with water and dried under high vacuum (0.111 g, 56%), mp 204–205 °C (heated slowly). The product **17** was homogeneous by TLC.

Anal. Calcd. for C₃₂H₃₈N₆O₈·1/2H₂O: C, 59.71, H, 6.11; N, 13.06. Found: C, 59.72; H, 5.98; N, 13.15.

8-[4-[[[[1-[[2-Aminoethyl]oxy]carbonyl]ethyl]amino]carbonyl]methylfoxy]phenyl]-1,3-dipropylxanthine Hydrobromide, 18—Compound **17** (26.6 mg, 42 $\times$ 10⁻⁶ mol) was dissolved with vortexing in 30% HBr/acetic acid (1 mL), and the solution was stirred at room temperature under nitrogen for 30 min. The flow rate of nitrogen was increased leaving an oil, and the product was precipitated as a microcrystalline solid from methanol/ether. The supernatant was removed with a Pasteur pipette, and the remaining white solid was washed (ether, 3 $\times$) and dried under high vacuum (25 mg, 100%), mp 273 $^{\circ}$ C dec. The product was homogeneous by TLC (R_f = 0.78, silica, using chloroform:methanol:acetic acid 10:10:1, by vol). MS: 501 ($M + 1$), 483, 329. ¹H NMR (DMSO-*d*₆) δ : 8.29 (t, 1H, CONH), 8.08 (d, 2H, J = 8.8 Hz, 8-ArH, ortho), 7.83 (br s, 2H, NH₂), 7.09 (d, 2H, J = 8.8 Hz, 8-ArH, meta), 4.57 (s, 2H, CH₂O), 4.20 (t, 2H, CH₂), 4.02, 3.87 (each, t, 2H, J = 7 Hz, Pr, CH₂), 3.40, 3.09 (each: m, 2H, CH₂), 2.57 (t, 2H, J = 6.9 Hz, CH₂), 1.74, 1.58 (each: q, 2H, Pr, CH₂), 0.89 (m, 2 $\times$ 3H, Pr, CH₃) ppm.

8-[4-[[[[1-[[2-[[3-Isothiocyanatophenyl]amino]thio]carbonyl]amino]ethyl]oxy]carbonyl]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3 dipropylxanthine, 19—Compound **19** was synthesized in 91% yield from compound **18** and *m*-phenylene diisothiocyanate by the general method given for compound **12**. The product was recrystallized from DMF/ether, was homogeneous by TLC (R_f = 0.84, silica, using chloroform:methanol:acetic acid 85:10:5, by vol), and reacted with ethylenediamine to form cleanly a new compound of lower R_f . ¹H NMR (DMSO-*d*₆) δ : 9.80 (s, 1H, ArNH), 8.23 (m, 2H, carbonyl-NH), 8.08 (d, 2H, 8-ArH, ortho), ArNCS: 7.60 (1H, H₆), 7.34 (2H, H_{2,4}), 7.15 (1H, H₃), 7.06 (d, 2H, 8-ArH, meta), 4.55 (s, 2H, CH₂O), 4.19 (m, 2H, CH₂), 4.01 (t, 2H, Pr, CH₂), 3.87 (t, 2H, Pr, CH₂), 3.73, 3.62, 3.15 (each: 2H, CH₂), 3.02, 2.87 (each: t, 2H, CH₂), 1.74, 1.58 (each: q, 2H, Pr, CH₂), 0.88 (m, 2 $\times$ 3H, Pr, CH₃) ppm.

8-[4-[[[[1-[[2-[[3-Isothiocyanato-5-[[2-[[3-(4-hydroxyphenyl)propionyl]amino]ethyl]amino]carbonyl]phenyl]-amino]thiocarbonyl]amino]ethyl]oxy]carbonyl]ethyl]amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine, 20—Compound **18** (8 mg, 14 μ mol) and 1-[[3-(4-hydroxyphenyl)propionyl]amino]-3,5-diisothiocyanatobenzene (ref 11, 19 mg, 45 μ mol) were dissolved in 1 mL of DMF, and diisopropylethylamine (5 μ L) was added with stirring. After 1 h, all of the amine (R_f = 0.08) had been consumed, as judged by TLC (silica, using chloroform:methanol: acetic acid 85:10:5, by vol). Ether was added, causing an oil to separate. The supernatant was removed, and the residue was crystallized from MeOH/ether. The solid was washed with ether and dried under vacuum to provide 8.6 mg of product (66% yield), which was homogeneous by TLC (R_f = 0.51, same system as above). ¹H NMR (DMSO-*d*₆) δ : 10.0 (s, 1H, ArNH), 8.7 (br s, 1H, OH), 8.59, 8.25 (each: t, 1H, NH), 8.08 (d, 2H, J = 8.8 Hz, 8-ArH, ortho), 7.94, 7.81 (each: m, 1H, NH), 7.76, 7.59 (each s, 1H, ArNCS), 7.07 (d, 2H, J = 8.8 Hz, 8-ArH, meta), 6.96, 6.64 (each, d, 2H, J = 8.3 Hz, ArOH), 4.55 (s, 2H, CH₂O), 4.19 (t, 2H, J = 5.3 Hz, CH₂), 4.01 (t, 2H, J = 6.9 Hz, Pr, CH₂), 3.86 (t, 2H, J = 7.3 Hz, Pr, CH₂), 3.74, 3.40, 3.25, 3.20 (each: m, 2H, CH₂), 2.68, 2.55, 2.29 (each: t, 2H, CH₂), 1.74, 1.58 (each: q, 2H, Pr, CH₂), 0.89 (m, 2 $\times$ 3H, Pr, CH₃) ppm.

Binding Assays

Bovine cerebral cortical membranes were prepared as described previously (12), from frozen brains obtained from Pel-Freez Biologicals Co. (Rogers, Arkansas). Membranes were treated with adenosine deaminase (0.5 U/mL) for 20 min at 37 $^{\circ}$ C prior to radioligand binding studies or incorporation studies.

Inhibition of binding of 1 nM [^3H]- N^6 -phenylisopropyl-adenosine or 0.2 nM [^3H]-8-cyclopentyl-1,3-dipropylxanthine (Dupont NEN, Boston, MA) to A_1 -adenosine receptors in bovine cerebral cortex membranes was assayed as described (13,14). Membranes (40 μg , 150 μL) were incubated for 1 h at 25 $^{\circ}\text{C}$ in a total volume of 1 mL, containing 100 μL of radioligand of the indicated concentration and 25 μL of the competing ligand (dissolved as a stock solution in DMSO). Samples of the drugs were dissolved freshly from solid and stored at -80°C . The DMSO solutions were diluted to a concentration of less than 0.1 mM prior to adding to aqueous medium. Bound and free radioligand were separated by addition of 4 mL of a solution containing 50 mM Tris hydrochloride, at pH 7.4 at 5 $^{\circ}\text{C}$, followed by vacuum filtration on glass filters with additional washes totaling 12 mL of buffer. Nonspecific binding was determined with 10 μM 2-chloroadenosine or 100 μM N^6 -cyclopentyladenosine. Protein was determined using the BCA protein assay reagents (Pierce Chemical Co., Rockford, IL).

All competition binding data was analyzed by nonlinear regression using the InPlot computer program (Graph-PAD, San Diego, CA), and IC_{50} values were converted to K_i values using the Cheng-Prusoff equation (15). The K_d values, used in these calculations for [^3H]PIA and [^3H]CPX binding to bovine brain A_1 -receptors were 130 ± 4 pM and 74 ± 3 pM, respectively (13).

To assay for irreversible inhibition, an incubation at 37 $^{\circ}\text{C}$ for 1 h in 50 mM Tris pH 7.4 with an isothiocyanate derivative was carried out and optionally followed by a second incubation in 50 mM Tris pH 7.4 with DTT or hydroxylamine to test for chemical reversibility. The incubation with DTT was always carried out at room temperature, and the incubation with hydroxylamine was either at rt or 37 $^{\circ}\text{C}$ (latter preferable). Washing cycles for inhibition experiments between incubations involved three cycles of centrifugation and resuspending the membrane pellet by stirring or homogenization using the Polytron. At the final step, prior to radioligand binding, the membranes were homogenized using a glass tissue grinder.

RESULTS

In order to demonstrate the conceptual approach of Figure 1, it was necessary to identify linkages (A–B in amine congener **1**) that may be cleaved chemically using reagents that are not detrimental to adenosine receptors and that would be compatible with an aqueous medium. Two likely possibilities were the reduction of disulfide bonds by thiols and the aminolysis of ester bonds by hydroxylamine. Receptors and other proteins, even those containing structurally important disulfide bridges, may be exposed to either thiol reagents or hydroxylamine below determined concentration limits and remain active (7).

The stability of adenosine receptors to potential cleavage conditions at room temperature was examined. The effects of chemical reagents on radioligand binding were measured in two different ways (Table 1): (1) as a preincubation with membranes followed by a washing step prior to radioligand binding and (2) addition of the reagent to the binding assay medium in the presence of the radioligand. Any difference between the two results might represent reversible competition by the highly concentrated chemical reagent for the radioligand binding site.

The stability of A_1 receptors to potential chemical cleavage conditions has not been reported. By analogy with a closely related protein sequence, our previous study of rabbit A_{2a} receptors demonstrated moderate stability of the receptor to 5 mM dithiothreitol (DTT) or 250 mM hydroxylamine (7). However, A_{2a} receptors potentially have more disulfide bridges (four have been proposed (24)) than do A_1 receptors; thus, it was necessary to test

the stability of A₁ receptors to these reagents. Bovine brain membranes containing A₁ adenosine receptors were exposed for 60 min to either a thiol or hydroxylamine and then washed and subjected to radioligand binding. It was observed that at a 5 mM concentration of DTT, the binding of [³H]CPX, an A₁-selective antagonist, and [³H]PIA, an A₁-selective agonist, was maintained near control levels (Table 1A). The stability of the receptor to hydroxylamine was even more striking, with a concentration of 250 mM tolerated.

The limits of stability at yet higher concentrations were probed (Table 1B). At room temperature, concentrations of hydroxylamine up to 3 M (maximum allowed by solubility) were well tolerated by the receptor, especially when [³H]CPX was used in the subsequent binding assay. Without an intervening wash, the IC₅₀ values for DTT inhibition of radioligand binding were 158 mM for [³H]-CPX and 85 mM for [³H]PIA binding. When DTT was removed prior to the binding assay, IC₅₀ values increased to 290 mM for [³H]CPX and 308 mM for [³H]PIA binding. The latter values more closely reflect the effects of DTT on the receptor protein, which likely contains disulfide linkages. When the hydroxylamine remained in the medium concurrently with the radioligand, agonist binding alone was adversely affected. The IC₅₀ value for hydroxylamine inhibition of [³H]PIA binding was 385 mM. Curiously, with an intervening wash, even a concentration of hydroxylamine of 2 M resulted in only 15% inhibition of [³H]PIA binding. This suggests that high concentrations of hydroxylamine interact noncovalently with a site on the receptor or on the radioligand that interferes with agonist binding alone. This complication notwithstanding, the use of hydroxylamine is acceptable in the scheme proposed in this study, because a washing step may be included and because an antagonist radioligand alone may suffice to demonstrate the feasibility of the cleavage scheme.

The next step was to identify purine derivatives that may be employed in this scheme. The previous trifunctional study (11) utilized a series of functionalized xanthine derivatives, based on the A₁ antagonist XAC (8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine), that acted as irreversible A₁-inhibitors at concentrations in the range of 10⁻⁷–10⁻⁶ M. We have also shown that several isothiocyanate derivatives of ADAC, an agonist with selectivity and subnanomolar affinity for A₁ adenosine receptors, acted as irreversible inhibitors of the receptor at concentrations in the range of 10⁻⁸ M. We reexamined the compounds *m*-DITC-XAC, **5**, and *m*-DITC-ADAC, **6**, as irreversible A₁-inhibitors in bovine brain membranes (Chart 1, Table 2). It is to be noted that these ligands are analogous to structure **3** in Figure 1 in which R = H, except that they are lacking the cleavable group A–B. A 1 h incubation with 100 nM *m*-DITC-XAC or with 10 nM *m*-DITC-ADAC resulted in inhibition of 80 or 48% of the [³H]CPX binding, respectively. This inhibition was not reversible upon repeated washing of the membranes.

It was necessary to demonstrate that the receptor inhibition was not reversible under the conditions intended to be employed for the cleavage step (i.e., exposure to DTT or hydroxylamine). If binding ability of the receptor were restored, as was found in a study of affinity labels for A_{2a}-adenosine receptors (7), it would indicate that the site of reaction between the isothiocyanate group and the receptor protein would be sensitive to these chemical reagents. The inhibition was stable, as summarized in Table 2. Neither DTT (50 mM) nor hydroxylamine (250 mM), present during a second incubation after removal of the affinity label, reversed this inhibition.

In the previous study of irreversible inhibitors of A₁-adenosine receptors (2) based on XAC and ADAC, it was observed that the chain length separating the pharmacophore and the reactive electrophilic group (an isothiocyanate) could be varied somewhat without loss of the irreversible binding feature of the ligand. Thus, it was reasonable that a chain extension

to include a cleavable linkage (A–B) would not preclude covalent binding to the A₁-receptor. The antagonist series, rather than the agonist series, was developed in the subsequent compounds to avoid ambiguity, since agonist binding is subject to modulation by the state of coupling between the receptor and G-protein. Also, the binding of the antagonist [³H]CPX is less sensitive than [³H]PIA binding to the presence of hydroxylamine. Thus, we designed several new amine congeners related to XAC, in which the terminal ethylenediamine moiety of the chain was extended by the CH₂ABCH₂ group placed in the middle. AB consisted of either SS (thiol cleavable), **11** (Figure 2), or COO (hydroxylamine cleavable), **18** (Figure 3). These amine congeners corresponded to structure **1** in the general scheme (Figure 1). Each amine congener was to be coupled to a bifunctional or trifunctional cross-linking reagent to form a potentially cleavable affinity label (structure **3**).

The amine congeners were synthesized from a xanthine carboxylic congener, **9** (XCC, 8-[4-[(carboxymethyl)oxy]-phenyl]-1,3-dipropylxanthine), which also served as an intermediate in the synthesis of XAC (**16**). The cysteamine (HS(CH₂)₂NH₂) conjugate of XCC, a thiol derivative, was prepared previously and found to be a potent adenosine antagonist (**17**). The K_i value of the cysteamine conjugate of XCC (**17**) was determined to be 16 nM vs [³H]PIA at rat A₁ receptors. That conjugate was also shown to be biologically active in a functional assay, in the inhibition of adenosine agonist-induced stimulation of adenylate cyclase via A_{2a}-receptors, with a K_B value was 76 nM (**17**). Cystamine (compound **7**) is a disulfide dimer of cysteamine. The conjugate of XCC and cystamine (compound **11**, Figure 2), an amine congener, was isolated previously as a byproduct in the synthesis of the cysteamine derivative. In this study compound **11** was synthesized by condensing XCC, **9**, with Boc-cystamine, **8**, followed by deprotection using TFA (Figure 2). The K_i value of the disulfide compound **11** was found to be 10 nM vs [³H]PIA at rat A_x receptors, indicating that chain extension was not detrimental to binding affinity.

The ester-containing amine congener (compound **18**, Figure 3) was synthesized by condensing XCC with the β-(Cbz-amino)ethyl ester of β-alanine, **16**, followed by deprotection using HBr/acetic acid.

The next step was to couple compounds **11** and **18** to diisothiocyanate derivatives and to show that the conjugates would irreversibly bind to bovine A₁ receptors. The amine derivatives reacted with *m*-phenylene diisothiocyanate to form compounds **12** and **19**, respectively. The K_i values for **12** and **19** in the displacement of [³H]CPX in a “competitive” assay were found to be 2.1 ± 0.26 and 9.7 ± 3.5 nM, respectively. Thus, receptor affinity has been preserved. In an assay of irreversible binding, both **12** and **19** were shown to be effective inhibitors (Tables 3 and 4), comparable in effectiveness to *m*-DITC-XAC, **5**.

The ability of DTT or hydroxylamine, present during a second incubation, to reverse this inhibition by compound **12** or **19**, respectively, was examined. Compound **12** at a concentration of 500 nM caused a loss of 63–69% of the radioligand binding (Table 3). This binding was partially restored (recovered 15–25% relative to control level) upon exposure to DTT. At 100 mM DTT the reversal of inhibition of the antagonist binding site was more effective than at 50 mM.

In Table 2 and in our previous study (2), 3-isobutyl-1-methylxanthine (IBMX) was added to the washing medium as a precaution for the removal of noncovalently bound xanthine. In the present study it was not necessary to wash the membranes overnight with IBMX. A comparison of the reversal of inhibition by compound **12** (preincubation at 500 nM) using DTT (10, 50 or 100 mM) overnight at room temperature, either in the presence or absence of IBMX (200 μM), gave identical results (data not shown).

Preincubation of bovine brain membranes with compound **19** (250 nM) caused a loss of 63% of the [³H]CPX binding (Table 4). This binding was partially restored upon exposure to hydroxylamine (100–500 mM). At 37 °C, 20% of the radioligand binding relative to control level was recovered, while at room temperature the recovery was less effective (Table 5). Varying the concentration of the isothiocyanate derivative did not improve the percent of subsequent recovery of binding.

Compound **20** was prepared from the amine congener, **18**, and a diisothiocyanate containing a (*p*-hydroxyphen-yl)propionyl group for radioiodination (**18**). This diisothiocyanate, corresponding to structure **2** (Figure 1) in which R = CONH(CH₂)₂NHCO(CH₂)₂PhOH, was reported previously as a trifunctional cross-linker (**11**). The *K_i* value for **20** in the displacement of [³H]CPX in a “competitive” assay was found to be 9.3 ± 2.0 nM. Preincubation of membranes with compound **20** at a concentration of 500 nM followed by washing caused a loss of 86 ± 2% (*n* = 6) of the [³H]CPX binding. This binding was partially restored (19 ± 3% of the fraction that was lost, *n* = 5) upon exposure to hydroxylamine (250 mM at 37 °C), reaching maximal regeneration after 1 h (Figure 4).

Temperature of incubation and pH were varied in an effort to improve the degree of recovery of binding following inhibition by compound **20**. The hydroxylamine incubation was compared at 37 °C for 1 h or at 25 °C overnight. The resulting regeneration of [³H]CPX binding was comparable in both cases. An overnight incubation of membranes at 37 °C increased the recovery (to 45%) but the binding in control membranes was diminished by 50% by 400 mM hydroxylamine. At 37 °C, an incubation with hydroxylamine for 1–2 h gave the highest degree of recovery of [³H]CPX binding. Incubations longer than 2 h resulted in the loss of binding in control membranes. The pH of the hydroxylamine medium (37 °C for 1 h) was varied from 6 to 10.5 (Table 6). Within the pH range of 7.4–9.5 the differences in recovery of [³H]CPX binding were minor, while outside of that range less binding was recovered.

DISCUSSION

A general approach for the reversible affinity labeling of receptors has been demonstrated for A₁-adenosine receptors. Two sequential steps of chemical modifications of the receptor protein, *i.e.*, affinity labeling and cleavage, result in a functionally-regenerated receptor protein (structure **4b**, Figure 1) that also contains a site for a reporter group (R). Such a reporter group may consist of a radioactive or spectroscopic label, and numerous possibilities have been explored in our previous studies of trifunctional reagents (**11**, **20**). The chain cleavage used in this study was chemically-induced, but as an alternative method photosensitive groups such as *o*-nitrobenzyl (**8**) may be included in protein-affinity labeling reagents.

The effects on the pharmacology of a portion of the cleaved ligand being left on the receptor following restoration of the radioligand binding is the subject of ongoing studies. Thus, the regenerated receptor may not be identical in binding properties to the native receptor.

The cleavable portion of the ligand consists of a xanthine amine congener, in which the pharmacophore and an amino group are separated by a cleavable, *i.e.*, disulfide or ester, linkage (compounds **11** and **18**, respectively). Cystamine, **7**, was previously incorporated into thiol-cleavable cross-linking reagents for oligonucleotides (**19**) and in biotin avidin probes (**10**). In those studies the disulfide bond was easily reduced in the presence of DTT. Similarly, hydroxylamine readily cleaves ester groups, and its ability to remove an affinity label from a fragment of the β-adrenergic receptor fragment was interpreted to indicate an ester linkage (**22**).

Use of these linkages in cleavable affinity labels assumes that the receptor itself is stable to the conditions needed for the cleavage reaction. In control experiments (Table 1) the native bovine A₁-receptor was not denatured by moderate concentrations of DTT or high concentrations of hydroxylamine. Millimolar concentrations of DTT denatured the A₁-receptor, presumably through reduction of protein disulfide bridges, as have been proposed in a receptor model (21). Nevertheless, somewhat selective reduction of the disulfide bond of the receptor-bound affinity label appears to have been accomplished in the concentration range of 0.05–0.1 M DTT. Hydroxylamine alone displaced agonist ([³H]PIA) binding from bovine A₁-receptors in a noncovalent manner. Perhaps hydroxylamine in its protonated form binds to the putative Na⁺ binding site on the second transmembrane helix of the A₁-receptor, identified by a consensus sequence (21). Binding at this site would be expected to affect agonist binding adversely, but not antagonist binding, consistent with the present findings.

Since the A₁-receptor was more stable to hydroxylamine than to DTT, it was the ester linkage that was selected for inclusion in a ligand, **20**, containing the iodinated (18) (*p*-hydroxyphenyl)propionyl group. This group has been used to incorporate an ¹²⁵I label in a xanthine that readily cross-links to purified A₁-receptors (20).

Isothiocyanate derivatives **12**, **19**, and **20** inhibited radioligand binding to A₁-receptors in bovine brain membranes in a manner that was not reversed by repeated washing. Similar behavior was observed for the binding of *m*-DITC-XAC, **5**, to bovine brain receptors, in which case covalent cross-linking was demonstrated by Western blot analysis (6). Hydroxylamine or DTT successfully restored binding of [³H]CPX in A₁-receptors inhibited by the appropriate cleavable xanthine isothiocyanate derivative. Binding was not fully restored, but the partial reversal is sufficient to illustrate the feasibility of this approach. It may be possible to purify the cleaved and functional receptor by affinity chromatography.

The A₁-receptor itself was stable to a wide pH range. Within this range, hydroxylamine reversed labeling by **20** by approximately 20% of the control value. Cleavage of an ester by hydroxylamine requires the free amine. At low pH, hydroxylamine is primarily protonated, which may explain why below pH 6.5 it was not effective.

The development of this approach for adenosine receptors may serve as a model for extending the method to derivatizing other G-protein coupled receptors, which have the same overall architecture, and conceivably to other biopolymers. A site-specifically labeled receptor that still binds ligand is potentially of use in the screening of drug analogs for affinity. A spectroscopic reporter group, such as a fluorescent label, present in a functional ligand binding site, may show sensitivity to ligand-bound and free states of the receptor. Thus, such a group may give a detectable signal that would report drug-receptor interactions in real time. Also, incorporation of a reactive handle, such as a thiol group, that likely results after DTT treatment of the A₁ receptor labeled by compound **12**, offers new possibilities for derivatizing receptors.

It may be possible to bind a functionalized receptor (e.g., bearing a free thiol group) to an affinity column. Binding of the receptor to an affinity support column may also be accomplished by immobilizing group R in a derivative similar to compound **20**, followed by the solubilized receptor and subsequently hydroxylamine. Such an immobilized receptor would have many envisioned uses, such as the determination of affinity of soluble ligands by retention on a flow through column. The biospecific elution of an adsorbed radioligand would indicate the presence of a high affinity competing ligand in solution. This scheme could potentially be used for screening libraries for active congeners (23).

LITERATURE CITED

- (1). Newman AH. Irreversible ligands for drug characterization. *Ann. Rep. Med. Chem.* 1990; 25:271–280.
- (2). Jacobson KA, Barone S, Kammula U, Stiles GL. Electrophilic derivatives of purines as irreversible inhibitors of A₁-adenosine receptors. *J. Med. Chem.* 1989; 32:1043–1051. [PubMed: 2709373]
- (3). Barrington WW, Jacobson KA, Stiles GL. Demonstration of distinct agonist and antagonist conformations of the A₁ adenosine receptor. *J. Biol. Chem.* 1989; 264:13157–13164. [PubMed: 2753906]
- (4). Curtis CA, Wheatley M, Bansal S, Birdsall NJ, Eveleigh P, Pedder EK, Poyner D, Hulme EC. Propylbenzylcholine mustard labels an acidic residue in transmembrane helix 3 of the muscarinic receptor. *J. Biol. Chem.* 1989; 264:489–495. [PubMed: 2909533]
- (5). Chorev M, Feigenbaum A, Keenan AK, Gilon C, Levitski A. *Eur. J. Biochem.* 1985; 146:9–14. [PubMed: 2981685]
- (6). Stiles GA, Jacobson KA. A new high affinity iodinated adenosine receptor antagonist as a radioligand/photoaffinity crosslinking probe. *Mol. Pharmacol.* 1987; 32:184–188. [PubMed: 3614192]
- (7). Jacobson KA, Stiles GL, Ji X-D. Chemical modification and irreversible inhibition of striatal A₂-adenosine receptors. *Mol. Pharmacol.* 42:123–133. [PubMed: 1635550]
- (8). Patchornik, A.; Jacobson, KA.; Strub, MP. Photo-reversible affinity labeling. In *Design and Synthesis of Organic Molecules Based on Molecular Recognition*. In: van Binst, G., editor. *Proceedings of the XVIIIth Solvay Conference on Chemistry*; Brussels. Springer; 1986. p. 235-241.
- (9). Denny JB, Blobel G. ¹²⁵I-labeled crosslinking reagent that is hydrophilic, photoactivatable, and cleavable through an azo linkage. *Proc. Natl. Acad. Sci. U.S.A.* 1984; 81:5286–5290. [PubMed: 6433347]
- (10). Haeussling L, Ringsdorf H, Schmitt FJ, Knoll W. Biotin-functionalized self-assembled monolayers on gold: surface plasmon optical studies of specific recognition reactions. *Langmuir.* 1991; 7:1837–1840.
- (11). Boring DL, Ji X-D, Zimmet J, Taylor KE, Stiles GL, Jacobson KA. Trifunctional agents as a design strategy for tailoring ligand properties: Irreversible inhibitors of A₁ adenosine receptors. *Bioconjugate Chem.* 1991; 2:77–88.
- (12). Chong PCS, Hodges RS. A new hetero-bifunctional cross-linking reagent for the study of biological interactions between proteins. *J. Biol. Chem.* 1981; 256:5064–5070. [PubMed: 7228869]
- (13). Garritsen, A. Ph.D. Thesis. Center for Bio-pharmaceutical Research; Leiden, Netherlands: 1990. *Molecular pharmacology of the adenosine A₁ receptor*; p. 58
- (14). Bruns RF, Fergus JH, Badger EW, Bristol JA, Santay LA, Hartman JD, Hays SJ, Huang CC. Binding of the A₁-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn Schmiedebergs Arch. Pharmacol.* 1987; 335:59–63. [PubMed: 3574492]
- (15). Cheng Y-C, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC₅₀) of an enzyme reaction. *Biochem. Pharmacol.* 1973; 22:3099–3108. [PubMed: 4202581]
- (16). Jacobson KA, Kirk KL, Padgett WL, Daly JW. Functionalized congeners of 1,3-dialkylxanthines: preparation of analogues with high affinity for adenosine receptors. *J. Med. Chem.* 1985; 28:1334–1340. [PubMed: 2993622]
- (17). Jacobson KA, de la Cruz R, Schulick R, Kiriasis L, Padgett W, Pfeleiderer W, Kirk KL, Neumeyer JL, Daly JW. 8-Substituted xanthines as antagonists as A₁ and A₂-adenosine receptors. *Biochem. Pharmacol.* 1988; 37:3653–3661. [PubMed: 3178879]
- (18). Bolton AE, Hunter WM. The labelling of proteins to high specific radioactivities by conjugation to a ¹²⁵I-containing acylating agent. *Biochem. J.* 1973; 133:529–539. [PubMed: 4733239]
- (19). Ferenz AE, Verdine GL. Disulfide cross-linked oligonucleotides. *J. Am. Chem. Soc.* 1991; 113:4000–4002.

- (20). Jacobson KA, Olah ME, Stiles GL. Trifunctional ligands: A radioiodinated high affinity acylating antagonist for the A₁ adenosine receptor. *Pharmacol. Commun.* 1992; 1:145–154.
- (21). van Galen PJM, Stiles GL, Michaels G, Jacobson KA. Adenosine A₁ and A₂ receptors: Structure-function relationships. *Med. Res. Rev.* 1992; 5:423–471. [PubMed: 1513184]
- (22). Eshdat Y, Chapot M-P, Strosberg AD. Chemical characterization of ligand binding site fragments from turkey β -adrenergic receptor. *FEBS Lett.* 1989; 246:166–170. [PubMed: 2540034]
- (23). Zuckermann RN, Kerr JM, Siani MA, Banville SC, Santi DV. Identification of highest-affinity ligands by affinity selection from equimolar peptide mixtures generated by robotic synthesis. *Proc. Nat. Acad. Sci. U.S.A.* 1992; 89:4505–4509.
- (24). Jacobson KA, van Galen PJM, Ji X.-d. Ramkumar V, Olah M, Stiles GL. Molecular Characterization of A₁ and A_{2a} adenosine receptors. *Drug Devel. Res.* 1993; 28:226–231. [PubMed: 23002320]

Figure 1.

General scheme for reversible affinity labeling that leaves a reporter group on a receptor protein. Structure **1** is an amine-functionalized congener that contains a cleavable (A–B) linkage. A conjugate, **3**, is formed with a trifunctional cross-linking reagent (structure **2**), which may be used to affinity label a receptor (as in **4a**). The A–B linkage is then cleaved (structure **4b**), which allows the free ligand containing the pharmacophore to diffuse away from the binding site.

Figure 2.

Synthesis of a thiol-cleavable xanthine amine congener, **11**, containing a disulfide linkage. The final step consisted of reaction with *m*-phenylene isothiocyanate to form **12**. Reagents: (a) di-*tert*-butyl dicarbonate; (b) EDAC/1-hydroxybenzotriazole, DMF; (c) TFA; (d) *m*-phenylene diisothiocyanate (R = H) or derivative thereof.

Figure 3.

Synthesis of a hydroxylamine-cleavable xanthine amine congener, **18**, containing an ester linkage. The final step consisted of reaction with *m*-phenylene isothiocyanate to form **19** or with 4-[2-[[[2-[[[3,5-diisothiocyanatobenzoyl]amino]ethyl]amino]carbonyl]-ethyl]-2-iodophenol to form **20**. Reagents: (a) EDAC/DMAP, DMF; (b) TFA; (c) EDAC/1-hydroxybenzotriazole, DMF; (d) HBr/acetic acid; (e) *m*-phenylene diisothiocyanate (R = H) or derivative thereof.

Figure 4.

Time course for the regeneration by hydroxylamine of the specific binding of [^3H]CPX to A_1 receptors (percent of initial control binding) following exposure of bovine brain membranes to compound **20**. The preincubation with **20** (500 nM) was carried out for 1 h at 37 °C. Following the standard washing procedure (3x), the membranes were treated with hydroxylamine (250 mM) for 1 h at 37 °C, and aliquots were removed at the times indicated and immediately diluted and washed 3 $\times$ in preparation for radioligand binding using 0.2 nM [^3H]CPX. The curve is from a single regeneration experiment, in which the binding at each time point was determined three times (mean $\pm$ s.e.m. shown).

Chart 1

Table 1

Stability of Bovine A₁-Adenosine Receptors to Reagents for Use in Cleavage Reaction: (A) at Fixed Concentration with Intervening Wash^a and (B) Concentration Dependence of Inactivation of Receptor Binding by Reagents for Potential Regeneration, with and without Intervening Wash^b

(A) reagent	specific binding remaining (% of control)	
	[³ H]CPX	[³ H]PIA
none	100	100
DTT (5 mM)	98 ± 3	97 ± 19
DTT (50 mM)	98 ± 2	98 ± 2
H ₂ NOH (250 mM)	96 ± 5	70 ± 8

(B) reagent	IC ₅₀ (mM) or % inhibition (at concn indicated)			
	[³ H]CPX		[³ H]PIA	
	wash: +	–	+	–
DTT	290 ± 37	158 ± 42	308 ± 17	85 ± 8
H ₂ NOH	0% (2M)	0% (3M)	15% (2 M)	385 ± 144

^aData are means ± SE of three to four experiments. Dithio-threitol or hydroxylamine was added during a 1 h preincubation with membranes at room temperature prior to radioligand binding. The concentrations of radioligand used were 0.2 and 1.0 nM for [³H]CPX and [³H]PIA, respectively.

^bData are means ± s.e.m. of four to five experiments. Dithiothreitol or hydroxylamine was added either (1) during a 1 h preincubation with membranes at room temperature and removed by washing or (2) during the radioligand binding. The concentrations of radioligand used were 0.2 and 1.0 nM for [³H]CPX and [³H]PIA, respectively.

Table 2

Inhibition of Bovine A₁-Adenosine Receptors by Purine Isothiocyanate Derivatives (without Cleavable Chains) and the Stability of this Inhibition to Reagents for Later Use in Cleavage Reaction^a

reagent	concn (mM)	inhibition (% of control)			
		<i>m</i> -DITC-XAC, 5	<i>m</i> -DITC-ADAC, 6	[³ H]CPX	[³ H]PIA
		[³ H]CPX	[³ H]PIA		
none		80 ± 4 (7)	55 ± 7 (3)	48 ± 8 (6)	51 ± 4 (4)
DTT	5	82 ± 4 (8)	55 ± 4 (4)	50 ± 4 (8)	52 ± 11 (4)
DTT	50	78.81	nd	52.58	nd
H ₂ NOH	250	78 ± 3 (9)	61 ± 8 (9)	47 ± 6 (6)	50 ± 12 (6)

^aData are means ± s.e.m. of three to nine experiments (*n* given in parentheses). After incubation with the isothiocyanate derivative (**5** or **6**) at 37 °C, membranes were washed three times, incubated overnight with IBMX (200 μM) in the presence of the indicated reagent, and again washed three times with IBMX and twice for DTT or hydroxylamine treatment. Radioligand binding was carried out with 0.2 nM [³H]CPX (**14**) or 1 nM [³H]PIA (**13**). nd: not determined.

Table 3Irreversible Inhibition of Radioligand Binding by Compound 12 and Reversal of the Inhibition by DTT^a

concn of DTT (mM)	³ H]CPX		³ H]PIA	
	% inhibn	% reversal	% inhibn	% reversal
0	63 ± 5		69 ± 5	
5	57 ± 5	6	52 ± 1	17
50	48 ± 4	15	44 ± 7	25
100	38 ± 4	25	40 ± 3	29

^aData are means ± s.e.m. of three to 11 experiments, expressed as percent of control binding. After incubation with the isothiocyanate derivative **12** (500 nM) for 1 h at 37 °C, membranes were washed three times, incubated with DTT for 60 min at room temperature, washed twice, and then incubated with 0.2 nM [³H]CPX (14) or 1 nM [³H]PIA (13).

Table 4

Relationship between Concentration of Compound 19 and the Ability of Hydroxylamine to Restore Binding of [³H]CPX

concn of 19 (nM)	inhibition of [³ H]CPX binding (% control)		reversal of inhibition (% control)
	(-H ₂ NOH)	(+H ₂ NOH)	
100	54.4	34.5	20
250	63.3 ± 1.2	43.6 ± 4.9	20
500	76.0 ± 3.0	54.0 ± 4.0	22

^aData are means ± s.e.m. of three experiments or a single experiment. After incubation with the isothiocyanate derivative at 37 °C for 1 h, membranes were washed three times, incubated with hydroxylamine (500 mM, 37 °C for 1 h), washed twice, and then incubated with 0.2 nM [³H]CPX (14). When the hydroxylamine incubation was carried out at 25 °C for 1 h, the degree of reversal was 8% (100 nM **19**), 9% (250 nM **19**), or 15% (500 nM **19**).

Table 5

Relationship between Temperature and the Regeneration by Hydroxylamine of [³H]CPX Binding Following Inhibition by Compound 19

concn of 19 (mM)	reversal of inhibition (% control)	
	37 °C	rt
250	24 ± 3	9 ± 5
500	23 ± 2	15, 15 (<i>n</i> = 2)

^aData are means ± s.e.m. of three experiments, unless noted. After incubation with the isothiocyanate derivative **19** at 37 °C for 1 h, membranes were washed three times, incubated with hydroxylamine (500 mM) at 37 °C for 1 h, washed three times, and then incubated with 0.2 nM [³H]CPX (14). Regeneration following overnight incubation with hydroxylamine at room temperature resulted in regeneration comparable to 1 h at 37 °C (data not shown).

Table 6

Relationship between pH and the Regeneration by Hydroxylamine of [³H]CPX Binding Following Inhibition by Compound 20

pH	reversal of inhibition (% control)
6.0	6
6.5	8
7.4	14 ± 3
8.5	13 ± 1
9.5	15 ± 3
10.5	9 ± 3

^aData are means ± s.e.m. of three experiments or the mean for two experiments. After incubation with the isothiocyanate derivative **20** at 37 °C for 1 h, membranes were washed three times, incubated with hydroxylamine (300 mM) for 1 h at 37 °C, washed three times, and then incubated with 0.2 nM [³H]CPX (14).