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Functionalized Congeners of 1,4-Dihydropyridines as Antagonist Molecular Probes for A₃ Adenosine Receptors

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Abstract

4-Phenylethynyl-6-phenyl-1,4-dihydropyridine derivatives are selective antagonists at human A₃ adenosine receptors, with K_i values in a radioligand binding assay vs [¹²⁵I]AB-MECA [N^6 -(4-amino-3-iodobenzyl)-5'- N -methylcarbamoyl-adenosine] in the submicromolar range. In this study, functionalized congeners of 1,4-dihydropyridines were designed as chemically reactive adenosine A₃ antagonists, for the purpose of synthesizing molecular probes for this receptor subtype. Selectivity of the new analogues for cloned human A₃ adenosine receptors was determined in radioligand binding in comparison to binding at rat brain A₁ and A_{2A} receptors. Benzyl ester groups at the 3- and/or 5-positions and phenyl groups at the 2- and/or 6-positions were introduced as potential sites for chain attachment. Structure–activity analysis at A₃ adenosine receptors indicated that 3,5-dibenzyl esters, but not 2,6-diphenyl groups, are tolerated in binding. Ring substitution of the 5-benzyl ester with a 4-fluorosulfonyl group provided enhanced A₃ receptor affinity resulting in a K_i value of 2.42 nM; however, a long-chain derivative containing terminal amine functionalization at the 4-position of the 5-benzyl ester showed only moderate affinity. This sulfonyl fluoride derivative appeared to bind irreversibly to the human A₃ receptor (1 h incubation at 100 nM resulting in the loss of 56% of the specific radioligand binding sites), while the binding of other potent dihydropyridines and other antagonists was generally reversible. At the 3-position of the dihydropyridine ring, an amine-functionalized chain attached at the 4-position of a benzyl ester provided higher A₃ receptor affinity than the corresponding 5-position isomer. This amine congener was also used as an intermediate in the synthesis of a biotin conjugate, which bound to A₃ receptors with a K_i value of 0.60 μ M.

INTRODUCTION

Medicinal chemists are currently developing agonists and antagonists that interact selectively with adenosine receptors, of which A₁, A_{2A}, A_{2B}, and A₃ subtypes are known (1). As the only subtype identified through cloning (2, 3), the A₃ adenosine receptor has demonstrated unique biological effects, SAR profile, and tissue distribution (4). Intense activation of A₃ receptors induces apoptosis (programmed cell death) (4). The A₃ receptor has also a cytoprotective action at low agonist concentrations (4). The first cytoprotective effects of an A₃ agonist [IB-MECA, N^6 -(3-iodobenzyl)-5'- N -methylcarbamoyl-adenosine] were shown following its chronic administration in gerbils in a model of stroke (5). The agonist was highly cerebroprotective and depressed nitric oxide synthase (5, 6). In cultured

chick cardiac myocytes, a brief prior exposure to nanomolar concentrations of the related 2-chloro derivative, Cl-IB-MECA, protected cells from damage induced by subsequent hypoxia (7), thus mimicking a phenomenon termed “preconditioning”. The protective effects of an A₃ receptor agonist on cardiac myocytes were also evident when added during the prolonged ischemia (8). A₃ receptor agonists at micromolar concentrations were found to induce apoptosis a variety of cell types, including tumor cell lines (9) and rat cardiac myocytes (10). Thus, the varied effects of A₃ receptor agonists appear to be dual and opposite, i.e., either cytoprotective or cytotoxic, depending on the level of receptor activation and the system studied.

A₃ adenosine receptor antagonists, although only recently introduced (11–16), were previously hypothesized to act as potential antiasthmatic (17), antiinflammatory (17), or cerebroprotective agents (18). The most promising leads for A₃ receptor antagonists have appeared recently among chemically diverse nonxanthine heterocycles (Figure 1). For example, the 1,4-dihydropyridines, that are potent blockers of L-type calcium channels (19, 20) and used widely in treating coronary heart disease, were found to bind with intermediate affinity to human A₃ receptors and consequently were structurally optimized for subtype selectivity (12). In the present and previous studies, we have used the 1,4-dihydropyridine (DHP) nucleus as a template for probing structure–activity relationships (SAR) at adenosine receptors. By careful structural modification, it has been possible to select for affinity at adenosine receptors and deselect for affinity at L-type Ca²⁺-channels.

The most potent antagonist of the human A₃ receptor yet reported is MRS 1220 (Figure 1, **1a**) (16), a derivative of the nonselective triazoloquinazoline antagonist CGS 15943. Within the series of DHP derivatives, affinity and selectivity for the human A₃ receptor were further enhanced in the trisubstituted analogue MRS 1191, 3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (Table 1, **1b**) (12). Other A₃ selective antagonists that have been recently reported include a flavonoid derivative (MRS 1067, **1c**) (11), a triazolophthalazine (L-249313, **1d**) (13), a pyridine (MRS 1523, **1e**) (14), and a pyridyl isoquinoline (VUF 8504, **1f**) (15).

In the present study, the SAR of 1,4-dihydropyridines as antagonist ligands for adenosine receptors has been expanded using a “functionalized congener approach” (21). By this approach, a chain substituent is attached to the pharmacophore at a strategic position, such that conjugation to other large molecules is made possible through an easily derivatized functional group, without losing the ability to bind to the receptor. This approach leads to increased flexibility of substitution and enhancement of potency/selectivity via distal interactions at the receptor. This approach has been developed for other selective adenosine receptor agonists and antagonists, such as the potent xanthine amine congener, XAC (8-[4-[[[(2-aminoethyl)-amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine) (34), and ligands for other receptors (35), leading to irreversibly binding affinity probes, fluorescent probes, etc.

A typical means of derivatization in the design of functionalized congeners is to locate a position on the pharmacophore that may readily accommodate substitution with a phenyl

¹Abbreviations: [¹²⁵I]AB-MECA, [¹²⁵I]N⁶-(4-amino-3-iodobenzyl)-5'-N-methylcarbamoyladenine; CGS 21680, 2-[4-[(2-carboxyethyl)phenyl]ethyl-amino]-5'-N-ethylcarbamoyl-adenosine, CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHO cells, Chinese hamster ovary cells; DHP, 1,4-dihydropyridine; DMAP, N,N-dimethylaminopyridine; DMSO, dimethyl sulfoxide; DPPA, diphenylphosphoryl azide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HEK cells, human embryonic kidney cells; IB-MECA, N⁶-(3-iodobenzyl)-5'-N-methylcarbamoyladenine; K_i, equilibrium inhibition constant; MRS 1097, 3,5-diethyl 2-methyl-6-phenyl-4-[2-phenyl-(E)-vinyl]-1,4-(±)-dihydropyridine-3,5-dicarboxylate; MRS 1163, N⁶-(3-isothiocyanatobenzyl)-5'-N-methylcarbamoyladenine; MRS 1191, 3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; NBS, N-bromosuccinimide; R-PIA, R-N⁶-phenylisopropyladenosine; SAR, structure–activity relationship; TBAF, tetrabutylammonium fluoride; TMS, tetramethylsilane; Tris, tris(hydroxymethyl)aminomethane.

group (34), without losing affinity for the receptor. In the present study, we have systematically explored the DHP template for additional sites of incorporation of phenyl substituents and various functional groups followed by chain extension. Fluorosulfonyl groups (29) were included in the analogues as an electrophilic group for potential covalent reaction with the receptor, which was indicated in binding experiments with one derivative. Finally, the incorporation of easily derivatizable amino groups at strategic positions was accomplished.

MATERIALS AND METHODS

Synthesis

Materials and Instrumentation—Phenylpropargyl aldehyde (25), benzyl acetoacetate (26a), ethyl benzoyl acetate (26b), 2-methoxyethyl acetoacetate (26i), 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (27), ethylene glycol (28d), 2-mercaptoethanol (28e), ethylene dithiol (28f), 2-trimethylsilylethanol (28h), *p*-nitrobenzyl alcohol (29), *p*-toluenesulfonyl fluoride (30a), *p*-toluenesulfonamide (30b), NBS, 2-(trimethylsilyl)ethanethiol, α -bromo-*p*-toluic acid, chloromethyl methyl ether, and 2-bromoethanol were purchased from Aldrich (St. Louis, MO). 2-Trimethylsilylethylthioethanol (28g) was prepared by reaction of 2-(trimethylsilyl)ethanethiol (1.1 mmol), 2-bromoethanol (1.0 mmol), and potassium hydroxide (1.5 mmol) in CH₃CN at room temperature for 4 h (yield, 78%). Compounds 1b–4 were prepared as described Jiang et al. (12). Dihydropyridines 21 [3-ethyl 5-[4-(2,2,2-trichloroethoxycarbonyl)]benzyl] 2-methyl-4-phenylethynyl-6-phenyl-1,4($\pm$)-dihydropyridine-3,5-dicarboxylate], 22 [3-ethyl 5-[4-(2-aminoethyl)aminocarbonylbenzyl]-2-methyl-4-phenylethynyl-6-phenyl-1,4($\pm$)-dihydropyridine-3,5-dicarboxylate], 1-(ethoxymethyl)-3-(ethoxycarbonyl)-2-methyl-4-(phenylethynyl)-6-phenyl-1,4-dihydropyridine-5-carboxylic acid (32b), compound 30c, and bromide 31c were prepared according to the method of Jiang et al. (12). Compounds 24a, 24b, and benzyl benzoyl acetate (26c) were prepared in our previous paper (14). All other materials were obtained from commercial sources.

Proton nuclear magnetic resonance spectroscopy was performed on a Varian GEMINI-300 spectrometer and all spectra were taken in CDCl₃. Chemical ionization (CI) mass spectrometry was performed with a Finnigan 4600 mass spectrometer and electron-impact (EI) mass spectrometry with a VG7070F mass spectrometer at 6 kV. Elemental analysis was performed either by Atlantic Microlab Inc. (Norcross, GA) or by Galbraith Laboratories, Inc. (Knoxville, TN).

General Procedure for Preparation of Substituted 1,4-Dihydropyridine (5–8a, 9–12, and 33, Scheme 1)

Equimolar amounts (0.5–1.0 mmol) of the appropriate β -enaminoester (24), aldehyde (25), and β -ketoester (26) were dissolved in 2–5 mL of absolute ethanol. The mixture was sealed in a Pyrex tube and heated, with stirring, at 80 °C for 18–24 h. After cooled to room temperature, the solvent was evaporated and the residue was purified by preparative TLC [silica 60; 1000 or 2000 μ m; Analtech, Newark, DE; petroleum ether-ethyl acetate (1:1–4:1)].

3,5-Dibenzyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4($\pm$)-dihydropyridine-3,5-dicarboxylate (5)—¹H NMR (CDCl₃): 2.34 (s, 3 H, 2-CH₃), 5.01 (AB, 2 H, *J* = 12.6 Hz, 3-OCH₂Ph), 5.24 (s, 1 H, 4-H), 5.27 (AB, 2 H, *J* = 13.8 Hz, 5-OCH₂Ph), 6.05 (s, br, 1 H, NH), 7.04–7.11 (m, 2 H, aromatic H's), 7.15–7.30 (m, 9 H, aromatic H's), 7.33–7.40 (m, 7

H, aromatic H's), 7.44–7.47 (m, 2 H, aromatic H's). MS (EI): m/z 539 (M^+), 448 ($M^+ - CH_2Ph$), 404 ($M^+ - CO_2CH_2Ph$), 91 ($^+CH_2Ph$, base).

3-Ethyl 5-Benzyl 4-phenylethynyl-2,6-diphenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (6)— 1H NMR ($CDCl_3$): 1.10 (t, 3 H, $J = 7.8$ Hz, 3- OCH_2CH_3), 4.16 (q, 2 H, $J = 7.8$ Hz, 3- OCH_2CH_3), 5.11 (s, 1 H, 4-H), 5.14 (AB, 2 H, $J = 12.9$ Hz, 5- OCH_2Ph), 6.86–8.10 (m, 21 H, NH and aromatic H's). MS (EI): m/z 539 (M^+), 448 ($M^+ - CH_2Ph$), 404 ($M^+ - CO_2CH_2Ph$), 91 ($^+CH_2Ph$, base).

3,5-Dibenzyl 4-Phenylethynyl-2,6-diphenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (7)— 1H NMR ($CDCl_3$): 5.07 (AB, 4 H, $J = 12.6$ Hz, 3- and 5- OCH_2Ph), 5.35 (s, 1 H, 4-H), 6.15 (s, br, 1 H, NH), 7.07–7.45 (m, 25 H, aromatic H's). MS (EI): m/z 601 (M^+), 510 ($M^+ - CH_2Ph$), 466 ($M^+ - CO_2CH_2Ph$), 91 ($^+CH_2Ph$, base). MS (CI- NH_3): m/z 619 ($M^+ + NH_4$), 602 ($M^+ + 1$). HRMS calcd: 601.2253. Found: 601.2255.

3-(2-Methoxyethyl) 5-Benzyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (8a)— 1H NMR ($CDCl_3$): 2.35 (s, 3 H, 2- CH_3), 3.37 (s, 3 H, 3- $OCH_2CH_2OCH_3$), 3.67 (s, 2 H, $J = 5.7$ Hz, 3- $OCH_2CH_2OCH_3$), 4.34 (m, 2 H, 3- $OCH_2CH_2OCH_3$), 5.01 (AB, 2 H, $J = 13.0$ Hz, 5- OCH_2Ph), 5.18 (s, 1 H, 4-H), 6.06 (s, br, 1 H, NH), 7.05–7.09 (m, 2 H, aromatic H's), 7.17–7.22 (m, 3 H, aromatic H's), 7.24–7.26 (m, 3 H, aromatic H's), 7.31–7.39 (m, 7 H, aromatic H's). MS (EI): m/z 507 (M^+), 448 ($M^+ - CH_3OCH_2CH_2$), 416 ($M^+ - CH_2Ph$), 372 ($M^+ - CO_2CH_2Ph$), 268 ($M^+ + 1 - CO_2CH_2Ph - CO_2CH_2CH_2OCH_3$, base). MS (CI- NH_3): m/z 525 ($M^+ + NH_4$), 508 ($M^+ + 1$).

3-(2-Methoxyethyl) 5-(4-Nitrobenzyl) 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (8b)—2-Methoxyethyl acetoacetate (**26i**, 160 mg, 1 mmol), phenylpropargyl aldehyde (**25**, 130 mg, 1 mmol), *p*-nitrobenzyl benzoyl acetate (**26j**, 299 mg, 1 mmol), and ammonium acetate (154 mg, 2 mmol) were dissolved in 3 mL of absolute ethanol. The mixture was stirred under N_2 at 95 °C for 24 h. After cooled to room temperature, the solvent was evaporated and the residue was purified by preparative TLC [silica 60; 1000 μ m; Analtech, Newark, DE; petroleum ether-ethyl acetate (1:1)] to give 127 mg (yield = 23%) of the product. 1H NMR ($CDCl_3$): 2.38 (s, 3 H, 2- CH_3), 3.40 (s, 3 H, 3- $OCH_2CH_2OCH_3$), 3.68 (m, 2 H, 3- $OCH_2CH_2OCH_3$), 4.37 (m, 2 H, 3- $OCH_2CH_2OCH_3$), 5.12 (AB, 2 H, $J = 13.5$ Hz, 5- OCH_2Ph), 5.20 (s, 1 H, 4-H), 6.04 (s, br, 1 H, NH), 7.17–7.44 (m, 12 H, aromatic H's), 8.02 (m, 2 H, aromatic H's). MS (CI- NH_3): m/z 570 ($M^+ + NH_4$), 553 ($M^+ + 1$), 282 ($M^+ - CH_3 - CO_2CH_2C_6H_4NO_2 - MeOCH_2CH_2O$, base).

3-(2-Hydroxyethyl) 5-Benzyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (9)— 1H NMR ($CDCl_3$): 2.35 (s, 3 H, 2- CH_3), 3.84 (m, 2 H, 3- OCH_2CH_2OH), 4.17 (m, 1 H, 3- OCH_2CH_2OH), 4.45 (m, 1 H, 3- OCH_2CH_2OH), 4.99 (AB, 2 H, $J = 12.6$ Hz, 5- OCH_2Ph), 5.17 (s, 1 H, 4-H), 6.20 (s, br, 1 H, NH), 7.02–7.39 (m, 15 H, aromatic H's). MS (EI): m/z 493 (M^+), 448 ($M^+ - HOCH_2CH_2$), 432 ($M^+ - HOCH_2CH_2O$), 404 ($M^+ - HOCH_2CH_2O_2C$), 402 ($M^+ - CH_2Ph$), 358 ($M^+ - CO_2CH_2Ph$), 91 ($^+CH_2Ph$, base).

3-(2-Mercaptoethyl) 5-Benzyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (10)— 1H NMR ($CDCl_3$): 1.77 (t, 1 H, $J = 8.7$ Hz, SH), 2.36 (s, 3 H, 2- CH_3), 2.82 (m, 2 H, 3- OCH_2CH_2SH), 4.23 (m, 1 H, 3- OCH_2CH_2SH), 4.42 (m, 1 H, 3- OCH_2CH_2SH), 5.01 (AB, 2 H, $J = 12.6$ Hz, 5- OCH_2Ph), 5.17 (s, 1 H, 4-H), 6.07 (s, br, 1 H, NH), 7.05–7.37 (m, 15 H, aromatic H's). MS (EI): m/z 509 (M^+), 448 ($M^+ -$

HSCH₂CH₂), 419 (MH⁺–CH₂Ph), 404 (M⁺ – HSCH₂CH₂O₂C), 374 (M⁺ – CO₂CH₂–Ph), 91 (⁺CH₂Ph, base).

3-(2-Mercaptoethylsulfanyl) 5-Benzyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (11)—¹H NMR (CDCl₃): 1.69 (t, 1 H, *J* = 8.7 Hz, SH), 2.36 (s, 3 H, 2-CH₃), 2.75 (dt, 2 H, *J* = 8.7, 6.9 Hz, 3-SCH₂CH₂SH), 3.20 (t, 1 H, *J* = 6.9 Hz, 3-SCH₂CH₂SH), 5.04 (AB, 2 H, *J* = 12.6 Hz, 5-OCH₂Ph), 5.32 (s, 1 H, 4-H), 6.14 (s, br, 1 H, NH), 7.05–7.41 (m, 15 H, aromatic H's). MS (EI): *m/z* 525 (M⁺), 432 (M⁺ – HSCH₂CH₂S), 404 (M⁺ – HSCH₂CH₂SOC), 330 (MH⁺–CO₂CH₂–Ph-SCH₂CH₂SH, base), 91 (⁺CH₂Ph). HRMS calcd for MH⁺: 526.1511. Found: 526.1466.

3-(2-Trimethylsilylethylthioethyl) 5-Benzyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (12)—¹H NMR (CDCl₃): 0.00 (m, 9 H, 3-SiCH₃), 0.85 (m, 2 H, 3-SCH₂CH₂SiMe₃), 2.34 (s, 3 H, 2-CH₃), 2.60 (m, 2 H, 3-SCH₂CH₂SiMe₃), 2.82 (m, 2 H, 3-CO₂CH₂–CH₂SCH₂CH₂SiMe₃), 4.33 (m, 2 H, 3-CO₂CH₂CH₂SCH₂–CH₂SiMe₃), 5.00 (AB, 2 H, *J* = 12.9 Hz, 5-OCH₂Ph), 5.15 (s, 1 H, 4-H), 6.03 (s, br, 1 H, NH), 7.04–7.40 (m, 15 H, aromatic H's). MS (CI–NH₃): *m/z* 609 (M⁺), 518 (M⁺ – CH₂Ph), 448 (M⁺ – CH₂CH₂SCH₂CH₂SiMe₃, base).

3-(2-Trimethylsilylethyl) 5-Ethyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (33)—¹H NMR (CDCl₃): 0.00 (m, 9 H, 3-SiCH₃), 0.99 (t, 3 H, *J* = 7.8 Hz, 5-OCH₂CH₃), 1.12 (t, 2 H, *J* = 8.8 Hz, 3-OCH₂CH₂SiMe₃), 2.57 (s, 3 H, 2-CH₃), 4.02 (m, 2 H, 3-CO₂CH₂CH₂SiMe₃), 4.33 (t, 2 H, *J* = 7.8 Hz, 5-OCH₂–CH₃), 5.13 (s, 1 H, 4-H), 5.87 (s, br, 1 H, NH), 7.25–7.44 (m, 10 H, aromatic H's).

General Procedure for Preparation of 1,4-Dihydropyridine-3,5-dicarboxylate Esters 13–15, 32c–d, and 19–20 (Scheme 3)

(Condition a) Compound **32a** or **32b** (0.04 mmol) was dissolved in dry acetone (2 mL) and anhydrous potassium carbonate (0.1 g), and the appropriate benzyl bromide (**31a–d**, 5 equiv.) was added, and the mixture was refluxed for 2–6 h. After filtering the mixture, the solvent was evaporated in vacuo. The residue was separated by preparative TLC (4:1 hexanes/EtOAc), and the desired *N*-protected 3- or 5-benzyl ester DHP was isolated.

(Condition b): Deprotection was achieved by dissolving these *N*-ethoxymethyl-protected DHPs in acetone (1 mL) and adding excess 1 N HCl (200 μL) and heating at 45 °C for 2–3 h. The solvent was evaporated followed by purification with preparative TLC (4:1 hexanes/EtOAc or 10:1 CHCl₃/MeOH) to afford the desired 3- or 5-benzyl ester DHP.

3-(4-Fluorosulfonylbenzyl) 5-Ethyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (13)—¹H NMR (CDCl₃): δ 0.93 (t, 3H, *J* = 6.84 Hz, 5-CH₂CH₃), 2.39 (s, 3H, 2-CH₃), 4.12 (q, 2H, *J* = 6.83 Hz, 5-OCH₂), 5.20 & 5.58 (AB, 2H, *J* = 14.65 Hz, 3-OCH₂), 5.21 (s, 1H, H-4), 6.23 (br, 1H, NH), 7.28–7.44 (m, 10H, 4-C₆H₅ and 6-C₆H₅), 7.71–7.89 (m, 4H, 3-C₆H₄).

3-(4-Sulfonylamidobenzyl) 5-Ethyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (14)—¹H NMR (CDCl₃): δ 0.93 (t, 3H, *J* = 6.84 Hz, 5-CH₂CH₃), 2.38 (s, 3H, 2-CH₃), 3.99 (m, 2H, 5-OCH₂), 4.77 (br, 2H, NH₂), 5.20 & 5.49 (AB, 2H, *J* = 13.67 Hz, 3-OCH₂), 5.19 (s, 1H, H-4), 5.97 (br, 1H, NH), 7.27–7.84 (m, 14H, 3-C₆H₄, 4-C₆H₅ and 6-C₆H₅). HPLC retention time: 7.66 min (> 95% purity) using solvent system 0.1 M TEAA (pH 5.0)/CH₃CN, 80:20 to 20:80, in 20 min with flow rate 1 mL/min.

3-[4-(2,2,2-Trichloroethoxycarbonyl)benzyl] 5-ethyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4(±)-dihydropyridine-3,5-dicarboxylate (15)—¹H NMR (CDCl₃): δ 0.95 (t, 3H, *J* = 6.84 Hz, 5-CH₂CH₃), 2.38 (s, 3H, 2-CH₃), 4.00 (m, 2H, 5-OCH₂), 4.96 (s, 2H, CH₂CCl₃), 5.22 and 5.51 (AB, 2H, *J* = 14.65 Hz, 3-OCH₂), 5.21 (s, 1H, H-4), 5.97 (br, 1H, NH), 7.25–8.07 (m, 14H, 3-C₆H₄, 4-C₆H₅ and 6-C₆H₅).

3-[4-(2-Aminoethyl)aminocarbonylbenzyl] 5-Ethyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4(±)-dihydropyridine-3,5-dicarboxylate (16)—Compound **15** (13.4 mg, 0.02 mmol) was mixed with ethylenediamine (0.175 mL). After stirring at room temperature for 4 h, the ethylenediamine was evaporated and the product purified by preparative TLC (5:1 CHCl₃/MeOH) to yield 7.0 mg of a white solid (59%). Compound **16** (*R*_f = 0.68, EtOAc) was shown to be pure by TLC.

¹H NMR (CDCl₃): δ 0.95 (t, 3H, *J* = 6.84 Hz, 5-CH₂CH₃), 2.37 (s, 3H, 2-CH₃), 2.78 (s, 2H, NH₂), 2.96 (t, 2H, *J* = 5.86 Hz, –CH₂–), 3.50 (m, 2H, –CH₂–), 4.00 (m, 2H, 5-OCH₂), 5.21 and 5.45 (AB, 2H, *J* = 13.68 Hz, 3-OCH₂), 5.20 (s, 1H, H-4), 5.92 (br, 1H, NH), 6.75 (br, 1H, –CH₂NH–), 7.27–7.73 (m, 14H, 3-C₆H₄, 4-C₆H₅ and 6-C₆H₅).

3-(4-Carboxybenzyl) 5-Ethyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4(±)-dihydropyridine-3,5-dicarboxylate (17)—Compound **32c** [3-(4-methoxyethoxycarbonylbenzyl) 5-ethyl 2-methyl-4-phenylethynyl-6-phenyl-1,4(±)-dihydropyridine-3,5-dicarboxylate] was prepared according to the general procedure using 30 mg of **32a** (0.067 mmol) and 86.8 mg of **31d** (0.335 mmol). The crude product was dissolved in 2 mL of acetone. To 1 mL of such acetone solution was added 0.2 mL of 1 N HCl. The mixture was stirred at 45 °C for 2 h. The solution was removed in vacuo and residue was purified by preparative TLC (silica 60; 1000 μM, 10:1 CHCl₃/MeOH) to give 13.2 mg of white solid, yield = 38%. Compound **17** (*R*_f = 0.17, MeOH) was shown to be pure by TLC.

¹H NMR (CDCl₃): δ 0.89 (t, 3H, *J* = 6.84 Hz, 5-CH₂CH₃), 2.38 (s, 3H, 2-CH₃), 4.01 (m, 2H, 5-OCH₂), 4.61 (s, 1H, OH), 5.21 and 5.49 (AB, 2H, *J* = 13.67 Hz, 3-OCH₂), 5.21 (s, 1H, H-4), 5.96 (br, 1H, NH), 7.35–8.04 (m, 14H, 3-C₆H₄, 4-C₆H₅ and 6-C₆H₅).

3-[4-(2-D-biotinylaminoethyl)aminocarbonylbenzyl] 5-Ethyl 2-Methyl-4-phenylethynyl-6-phenyl-1,4(±)-dihydropyridine-3,5-dicarboxylate (18)—Compound **16** (6.1 mg, 0.01 mmol) and *N*-hydroxysuccinimide biotin (4.4 mg, 0.01 mmol) were dissolved in 0.5 mL of anhydrous DMF and stirred at room temperature overnight. The solvent was evaporated and the product was purified by preparative TLC [12:1 CHCl₃/MeOH followed by 6:1 CHCl₃/MeOH] to yield 5.6 mg of a yellow solid (64%). Compound **18** (*R*_f = 0.73, MeOH/EtOAc = 1/3) was shown to be pure by TLC.

¹H NMR (CDCl₃): δ 0.90 (t, 3H, *J* = 6.84 Hz, 5-CH₂CH₃), 1.32 (br, 1H, H-4'), 1.60 (t, 1H, *J* = 6.84 Hz, H-2'), 1.74 (br, 1H, H-3'), 2.17 (m, 4H, 2 X –CH₂–), 2.37 (s, 3H, 2-CH₃), 2.63 (d, 2H, *J* = 12.70 Hz, –CH₂–5'), 2.82 (m, 2H, –CH₂–), 2.97 (m, 2H, –CH₂–), 3.47 (br, 1H, NH), 3.55 (br, 1H, NH), 3.94 (q, 2H, *J* = 6.84 Hz, 5-OCH₂), 4.12 (br, 2H, –CH₂–), 4.39 (br, 2H, –CH₂–), 5.16 (s, 1H, H-4), 5.21 and 5.40 (AB, 2H, *J* = 13.67 Hz, 3-OCH₂), 5.60 (br, 1H, NH), 6.29 (br, 1H, NH), 6.54 (br, 1H, NH), 7.16–7.77 (m, 14H, 3-C₆H₄, 4-C₆H₅ and 6-C₆H₅).

3-Ethyl 5-(4-Fluorosulfonylbenzyl) 2-Methyl-4-phenylethynyl-6-phenyl-1,4(±)-dihydropyridine-3,5-dicarboxylate (19)—¹H NMR (CDCl₃): δ 1.36 (t, 3H, *J* = 6.83 Hz, 3-CH₂CH₃), 2.39 (s, 3H, 2-CH₃), 4.30 (m, 2H, 3-OCH₂), 5.04 and 5.13 (AB, 2H, *J* =

14.66 Hz, 5-OCH₂), 5.19 (s, 1H, 4-H), 6.32 (br, 1H, NH), 7.23–8.00 (m, 14H, 4-C₆H₅, 5-C₆H₄, and 6-C₆H₅).

3-Ethyl 5-(4-Sulfonamidobenzyl) 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (20)—¹H NMR (CDCl₃): δ 1.35 (t, 3H, *J* = 6.84 Hz, 3-CH₂CH₃), 2.38 (s, 3H, 2-CH₃), 4.29 (m, 2H, 3-OCH₂), 4.74 (br, 2H, NH₂), 5.10 and 5.18 (AB, 2H, *J* = 13.68 Hz, 5-OCH₂), 5.18 (s, 1H, 4-H), 5.93 (br, 1H, NH), 7.16–7.83 (m, 14H, 4-C₆H₅, 5-C₆H₄, and 6-C₆H₅). HPLC retention time: 7.42 min (>95% purity) using solvent system 0.1 M TEAA (pH 5.0)/CH₃CN, 80:20 to 20:80, in 20 min with flow rate 1 mL/min.

3-Ethyl 5-(4-Carboxybenzyl) 2-Methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (23)—Compound **32d** [3-ethyl 5-(4-methoxyethoxycarbonylbenzyl) 2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate] was prepared in quantitative yield according to the general procedure using 35 mg of **32b** (0.0786 mmol) and 102 mg of **31d** (0.392 mmol). An acetone solution (1 mL) of 25.3 mg (0.041 mmol) of **32d** was mixed with 1 N HCl (0.15 mL), and the mixture was stirred at 45 °C for 2 h. The solution was removed in vacuo and residue was purified by preparative TLC (silica 60; 1000 μM, 10:1 CHCl₃/MeOH) to give 10.9 mg of light yellow solid, yield) 51%.

¹H NMR (CDCl₃): δ 1.34 (t, 3H, *J* = 6.84 Hz, 3-CH₂CH₃), 2.37 (s, 3H, 2-CH₃), 4.26 (m, 2H, 3-OCH₂), 5.00 & 5.15 (AB, 2H, *J* = 13.68 Hz, 5-OCH₂), 5.17 (s, 1H, 4-H), 5.99 (br, 1H, NH), 7.07–7.92 (m, 14H, 4-C₆H₅, 5-C₆H₄, and 6-C₆H₅).

Synthesis of β-Ketoesters **26d–h** (Scheme 2)

β-Ketoesters **26d–h** were prepared by the reaction of 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (**27**) and an alcohol or a thiol (eq 1 in Scheme 2). Equimolar amounts (for example, 3 mmol) of compound **27** and an alcohol or a thiol were heated with a little toluene (1–2 mL) at 80 °C in a sealed tube overnight. After cooled to room temperature, the solvent was removed under reduced pressure and the residue was chromatographed to give desired products in satisfactory yields (57% for **26d**, 39% for **26e**, 39% for **26f**, 95% for **26g**, 95% for **26h**).

2-Hydroxyethyl Acetoacetate (26d)—¹H NMR (CDCl₃/TMS): δ 2.29 (s, 3 H, CH₃CO), 2.43 (s, br, 1 H, OH), 3.54 (s, 2 H, CH₃COCH₂CO), 3.84 (m, 2 H, CO₂CH₂CH₂OH), 4.31 (t, *J* = 4.8 Hz, 2 H, CO₂CH₂CH₂OH). MS (CI/NH₃): 164 (M⁺ + NH₄, base), 147 (M⁺ + 1), 129 (M⁺ – OH).

2-Mercaptoethyl Acetoacetate (26e)—¹H NMR (CDCl₃/TMS): δ 1.54 (t, *J* = 8.7 Hz, 1 H, SH), 2.29 (s, 3 H, CH₃CO), 2.78 (dt, *J* = 8.7, 6.9 Hz, 2 H, CO₂CH₂CH₂SH), 3.50 (s, 2 H, CH₃COCH₂CO), 4.28 (t, *J* = 6.9 Hz, 2 H, CO₂CH₂CH₂SH). MS (CI/NH₃): 180 (M⁺ + NH₄, base).

S-(2-Mercaptoethyl) 3-Oxothiobutyrate (26f)—¹H NMR (CDCl₃/TMS): δ 1.64 (t, *J* = 8.7 Hz, 1 H, SH), 2.28 (s, 3 H, CH₃CO), 2.74 (dt, *J* = 8.7, 6.9 Hz, 2 H, COSCH₂CH₂SH), 3.16 (t, *J* = 6.9 Hz, 2 H, COSCH₂CH₂SH), 3.70 (s, 2 H, CH₃COCH₂CO). MS (CI/NH₃): 196 (M⁺ + NH₄, base), 179 (M⁺ + 1).

(2-Trimethylsilylethylthio)ethyl Acetoacetate (26g)—¹H NMR (CDCl₃/TMS): δ 0.03 (s, 9 H, SiMe₃), 0.86 (m, 2 H, Me₃SiCH₂CH₂S), 2.28 (s, 3 H, CH₃CO), 2.60 (m, 2 H, Me₃-SiCH₂CH₂S), 2.78 (t, *J* = 6.8 Hz, 2 H, SCH₂CH₂O₂C), 3.48 (s, 2 H,

$\text{CH}_3\text{COCH}_2\text{CO}$), 4.29 (t, $J = 6.8$ Hz, 2 H, $\text{CO}_2\text{CH}_2\text{-CH}_2\text{S}$). MS (CI/ NH_3): 280 ($\text{M}^+ + \text{NH}_4$), 161 ($\text{M}^+ - \text{CH}_2\text{-CH}_2\text{SiMe}_3$).

2-Trimethylsilylethyl Acetoacetate (26h)— ^1H NMR (CDCl_3/TMS): δ 0.03 (s, 9 H, SiMe_3), 1.01 (m, 2 H, $\text{Me}_3\text{-SiCH}_2\text{CH}_2$), 2.26 (s, 3 H, CH_3CO), 3.43 (s, 2 H, $\text{OCCH}_2\text{-CO}$), 4.22 (m, 2 H, $\text{Me}_3\text{SiCH}_2\text{CH}_2\text{O}$).

Preparation of *p*-Nitrobenzyl Benzoyl Acetate (26j) (eq 2 in Scheme 2)

Ethyl benzoyl acetate (**26b**, 1.92 g, 10 mmol) and *p*-nitrobenzyl alcohol (**29**, 1.53 g, 10 mmol) in toluene (10 mL) were heated with stirring for 24 h. The solvent was removed, and the residue was chromatographed [silica 60, petroleum ether-ethyl acetate (2:1)] to give **26j** (2.08 g, yield) 82%). ^1H NMR (CDCl_3/TMS): δ 4.11 (s, 2 H, PhCOCH_2CO), 5.30 (s, 2 H, $\text{CH}_2\text{C}_6\text{H}_4\text{NO}_2\text{-p}$), 7.47–7.65 (m, 5 H, aromatic H's), 7.94 (d, $J = 9.0$ Hz, 2 H, aromatic H's), 8.20 (d, $J = 9.0$ Hz, 2 H, aromatic H's). MS (CI/ NH_3): 317 ($\text{M}^+ + \text{NH}_4$, base), 300 ($\text{M}^+ + 1$).

Preparation of Bromides 31a and 31b (Scheme 3) (17)

A solution of *p*-toluenesulfonyl fluoride (**30a**, 1.0 equiv) or *p*-toluenesulfonamide (**30b**, 1.0 equiv), *N*-bromosuccinimide (1.0 equiv), and a catalytic amount of benzoyl peroxide dissolved in benzene (for example, 6 mmol of NBS using 10 mL of benzene) was stirred at 60 °C for 1 h. After the mixture cooled, the succinimide was filtered off and the filtrate chromatographed with pre parative TLC to yield the desired products (yield) 65% for **31a** and 62% for **31b**).

p-(Bromomethyl)benzenesulfonyl Fluoride (31a)— ^1H NMR (CDCl_3/TMS): δ 4.52 (s, 2 H, CH_2), 7.65 (d, $J = 7.8$ Hz, 2 H, aromatic H's), 8.00 (d, $J = 7.8$ Hz, 2 H, aromatic H's).

(p-Bromomethyl)benzenesulfonamide (31b)—MS (EI): 249 and 251 (M^+).

Preparation of Methoxymethyl 4-(Bromomethyl)-benzoate (31d) (Scheme 3)

α -Bromo-*p*-toluic acid (3.37 g, 15.7 mmol) was dissolved in DMF (10 mL). Chloromethyl methyl ether (1.26 g, 1.19 mL, 15.7 mmol) was then added, followed by adding triethylamine (1.9 g, 2.62 mL, 18.8 mmol). The reaction was stirred at room temperature overnight. Water (10 mL) was added, and the mixture was extracted with dichloromethane, washed with water, brine, and dried over MgSO_4 . Column chromatography (hexanes/ethyl acetate = 5/1) gave 0.588 g of light yellowish oil, yield = 15%. ^1H NMR (CDCl_3/TMS): δ 3.55 (s, 3 H, OCH_3), 4.62 (s, 2 H, BrCH_2), 5.50 (s, 2 H, OCH_2O), 7.48 (d, $J = 7.8$ Hz, 2 H, aromatic H's), 8.08 (d, $J = 7.8$ Hz, 2 H, aromatic H's). MS (EI): 259 (M^+).

Preparation of 1-Ethoxymethyl-2-methyl-5-ethoxycarbonyl-4-phenylethynyl-6-phenyl-1,4-($\pm$)-dihydropyridine-3-carboxylic acid (32a) (Scheme 4)

Sodium hydride (60% in mineral oil, 107 mg, 4.46 mmol) was added to compound **33** in solution of DMF (10 mL). The mixture was stirred for 30 min, chloromethyl ethyl ether (422 mg, 0.414 mL, 4.46 mmol) was added slowly to the solution under N_2 at room temperature and stirred for 2h. The reaction was quenched by adding cold water (10 mL), extracted with ethyl acetate (10 mL $\times$ 2), the organic layer was washed with water (10 mL $\times$ 2), brine (10 mL $\times$ 2), dried with sodium sulfate. The solvent was evaporated and residue was purified with preparative TLC (silica gel 60, hexanes/EtOAc: 4/1) to give N-protected product **34** 497 mg, yield = 49%. ^1H NMR (CDCl_3): 0.00 (m, 9 H, 3-Si CH_3), 0.88 (t, 3 H, $J = 6.8$ Hz, 5- CH_2CH_3), 0.95 (t, 2 H, $J = 6.8$ Hz, 1- OCH_2CH_3), 1.12 (t, 2 H, $J = 8.8$ Hz, 3- $\text{OCH}_2\text{CH}_2\text{SiMe}_3$), 2.62 (s, 3 H, 2- CH_3), 3.15 (m, 1 H, 1- OCH_2CH_3), 3.69 (m, 1 H, 1- OCH_2CH_3), 3.92 (q, 2 H, $J = 6.8$ Hz, 5- OCH_2CH_3), 4.33 (t, 2 H, $J = 6.8$ Hz, 3-

$\text{CO}_2\text{CH}_2\text{CH}_2\text{SiMe}_3$), 4.47 and 4.88 (AB, 2 H, $J = 10.7$ Hz, $\text{N-CH}_2\text{O}$), 5.09 (s, 1 H, 4-H), 7.24–7.42 (m, 10 H, aromatic H's). MS (CI- NH_3): m/z 563 ($\text{M}^+ + \text{NH}_4$), 546 ($\text{M}^+ + 1$).

TBAF (hydrate, 2.49 mmol, 1 M solution in THF) was added to a solution of **34** (340 mg, 0.623 mmol) in DMF (2 mL). The mixture was stirred under N_2 at room temperature for 2 h, diluted with ethyl acetate (20 mL), washed with 1 N HCl (5 mL), H_2O (20 mL $\times$ 2) and brine (20 mL $\times$ 2), and dried with magnesium sulfate. The solvent was evaporated, and residue was separated with preparative TLC to give 271 mg of compound **32a** as a yellow solid, yield = 98%. ^1H NMR (CDCl_3): δ 0.95 (m, 6 H, OCH_2CH_3), 2.67 (s, 3H, 2- CH_3), 3.16 and 3.69 (m, 2 H, $\text{N-CH}_2\text{OCH}_2\text{CH}_3$), 3.94 (q, 2 H, $J = 6.8$ Hz, 5- OCH_2), 4.50 and 4.90 (AB, 2 H, $\text{N-CH}_2\text{O}$), 5.13 (s, 1 H, 4-H), 7.18 (br, 1 H, COOH), 7.24–7.43 (m, 10 H, 2 $\times$ C_6H_5). MS (EI): m/z 445 (M^+).

Pharmacology. Radioligand Binding Studies—Binding of [^3H] $R\text{-N}^6$ -

phenylisopropyladenosine ([^3H] $R\text{-PIA}$) to A_1 receptors from rat cerebral cortex membranes and of [^3H]-2-[4-[(2-carboxyethyl)phenyl]ethylamino]-5'- N -ethylcarbamoyladenosine ([^3H]CGS 21680) to A_{2A} receptors from rat striatal membranes was performed as described previously (22, 23). Adenosine deaminase (3 units/mL) was present during the preparation of the brain membranes, in a preincubation of 30 min at 30 °C and during the incubation with the radioligands.

Binding of [^{125}I] N^6 -(4-amino-3-iodobenzyl)-5'- N -methylcarbamoyladenosine ([^{125}I]AB-MECA) to membranes prepared from HEK-293 cells stably expressing the human A_3 receptor (2), clone HS-21a (Receptor Biology, Inc., Beltsville, MD) or to membranes prepared from CHO cells stably expressing the rat A_3 receptor was performed as described (11, 24). The assay medium consisted of a buffer containing 10 mM Mg^{2+} , 50 mM Tris, and 1 mM EDTA, at pH 8.0. The glass incubation tubes contained 100 μL of the membrane suspension (0.3 mg of protein/mL, stored at -80 °C in the same buffer), 50 μL of [^{125}I]AB-MECA (final concentration 0.3 nM), and 50 μL of a solution of the proposed antagonist. Nonspecific binding was determined in the presence of 100 μM N^6 -phenylisopropyladenosine ($R\text{-PIA}$).

All nonradioactive compounds were initially dissolved in DMSO and diluted with buffer to the final concentration, where the amount of DMSO never exceeded 2%.

Incubations were terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester (Brandell, Gaithersburg, MD). The tubes were rinsed three times with 3 mL of buffer each.

At least five different concentrations of competitor, spanning 3 orders of magnitude adjusted appropriately for the IC_{50} of each compound, were used. IC_{50} values, calculated with the nonlinear regression method implemented in the Prism program (Graph-PAD, San Diego, CA), were converted to apparent K_i values using the Cheng–Prusoff equation (25) and K_d values of 1.0 and 14 nM for [^3H] $R\text{-PIA}$ and [^3H]CGS 21680, respectively, and 0.59 nM for binding of [^{125}I]AB-MECA at human A_3 receptors, respectively.

For studies of irreversible binding, membranes were incubated with the indicated (Figure 2) concentration of ligand for 1 h at RT. Membranes were then washed 10 times by sequential resuspension and centrifugation with buffer (50 mM Tris, 10 mM Mg^{2+} , and 1 mM EDTA) at pH 7.4 containing 0.02% CHAPS. Adenosine deaminase (3 units/mL) was present during all steps. The final pellet was suspended in a fixed volume of the above buffer solution for the A_3 receptor binding assay as described.

RESULTS

Synthesis

The structures of the 1,4-dihydropyridines and related derivatives (**1b–23**) tested for affinity in radioligand binding assays at adenosine receptors are shown in Table 1. As in the previous studies (12, 14), the basic synthesis of the DHP nucleus consisted of the Hantzsch condensation (Scheme 1, reviewed in ref 19). This method involves a three component reaction of a 3-amino-2-propenoate ester, such as benzyl 3-amino-3-phenyl-2-propenoate (Scheme 1), **24a**, an aldehyde, such as phenylpropargyl aldehyde, **25**, and a β -ketoester, such as benzyl acetoacetate, **26a**, that were dissolved in ethanol and refluxed. β -Ketoesters containing free hydroxyl and thio groups, **26d–f**, for use in the unprotected form in the condensation reaction, were synthesized as shown (Scheme 2). A silyl-protected thiol, **26g**, was also included.

The synthesis of DHP derivatives containing benzyl esters at the 3- and 5-positions, that were substituted with various acyl and sulfonyl groups, some of which were chemically reactive, was best approached through the alkylation of a free carboxylic acid using the corresponding benzyl bromide (Scheme 3). The benzyl bromides, **31a–c**, were synthesized by bromination of the corresponding toluene derivatives, **30a–c**, using *N*-bromosuccinimide. Use of compounds **30c** and **31c** in this context was described previously (12). The appropriate 3- or 5-carboxy derivative of the 6-phenyl-4-phenylethynyl-DHP was prepared by the methodology described (for the 5-carboxy derivative) in Jiang et al. (12). This approach incorporates a protecting group, a (2-trimethylsilylethyl)ester, at the 5-ester (12) or 3-ester (Scheme 4) position, that allows for facility of substitution of this ester and protection of the 1-position with the ethoxymethyl group (12). The 5-(2-trimethylsilylethyl)ester group was introduced at the stage of the Hantzsch condensation and subsequently removed using TBAF to provide **32b**. The resulting free carboxylic acid was then esterified in high yield by means of alkylation using the appropriate benzyl bromide, **31**, with potassium carbonate in acetone. The 1-ethoxymethyl group was finally removed upon heating at 45 °C for 1 h with 1 N HCl in acetone, to provide 5-position substituted benzyl esters, **19–21**, or 3-position esters, **13–15**.

Several additional modifications of functionality at the p-position of these benzyl esters were carried out. The 2,2,2-trichloroethyl derivatives (12), **15** and **21** (Scheme 3 and Table 1), were aminolyzed selectively using neat ethylenediamine at room temperature, to provide the amine functionalized congeners, **16** and **22**, respectively. As demonstrated by Jiang et al. (12), the ester groups at the 3- and 5-positions of the DHP were not susceptible to reaction with ethylenediamine under these conditions. Also, the methoxymethyl groups of **33** and **34** were removed using 1 N HCl to provide the corresponding carboxylic acid derivatives, **17** and **23** (Table 1). One of the primary amine congeners, **16**, was also acylated using a biotin-active ester to provide the biotin conjugate, **18**.

Pharmacology

Compounds **2** and **3** are 3,5-diethyl ester derivatives of 6-phenyl-1,4-dihydropyridines previously shown to act as selective A_3 receptor antagonists. Furthermore, the incorporation of a single benzyl ester at the 3- or 5-position was shown to enhance selectivity for the A_3 subtype. Several related benzyl esters, **4** and **5**, were included. As previously reported, the 3-benzyl ester, **4**, is >600-fold selective, although not as potent as the 3,5-diethyl ester, **3**, while the 5-benzyl ester, **1b**, is twice as potent at A_3 receptors as **3**. Combining both benzyl esters, in **5**, did not alter the receptor affinity of the 3-benzyl ester, **4**. However, at the 2- and 6-positions, double substitution with phenyl groups, as in compounds **6** and **7**, prevented binding to adenosine receptors. Transformation of the 3-ethyl ester of **1b** into a β -

methoxyethyl ester, **8a**, enhanced affinity at A₃ receptors by ~15-fold. Incorporation of a p-nitro group in the 5-benzyl ester substituent, **8b**, which was shown previously to enhance A₃ receptor affinity by roughly an order of magnitude (12), had no further enhancing effect on the affinity of the β-methoxyethyl ester. Incorporation of a β-hydroxyl group in the 3-ethyl ester substituent, **9**, had an affinity-enhancing effect at A₃ adenosine receptors. Curiously, a thiol group at the same position was much less favorable for affinity at A₃ receptors, both for the 3-ester analogue, **10**, and the corresponding 3-thioester, **11**. A β-trimethylsilylethyl derivative, **12**, only weakly bound to adenosine A₃ receptors.

The structure activity relationships of p-substituted 3- and 5-ester positions were probed, as potential sites for attachment of functionalized chains. Ring substitution of the 5-benzyl ester with a 4-fluorosulfonyl group, **19**, provided enhanced selectivity of >30000-fold for the A₃ receptor, while similar substitution of the corresponding 3-benzyl ester, **13**, was not affinity enhancing. The corresponding sulfonamides, **14** and **20**, displayed only moderate potency and selectivity, with only minor affinity differences between 3- and 5-isomers. The corresponding 3- and 5- carboxylates, **17** and **23**, were prepared, and only **17** bound to A₃ receptors with moderate potency. A DHP containing a long amine-functionalized chain attached at the 3-position, **16**, displayed moderate affinity, while the corresponding 5-isomer, **22**, was 5-fold less potent at A₃ receptors and nonselective.

Since an amine-functionalized chain attached at the 4-position of a benzyl ester at the 3-position of the DHP ring provided higher affinity A₃ receptors, **16** was used as an intermediate in the synthesis of a biotin conjugate, **18**. The biotin derivative showed >100-fold selectivity for human A₃ receptors.

Unlike related derivatives not containing a reactive, electrophilic group, the sulfonyl fluoride derivative, **19**, appeared to irreversibly bind to the A₃ receptor in a concentration-dependent manner (Figure 2). A 1 h preincubation with this derivative resulted in the loss of binding in a subsequent experiment following exhaustive washing of the membranes. At 100 nM **19**, approximately 56% of human A₃ receptor radioligand binding sites in transfected HEK-293 cell membranes were irreversibly inhibited. As control experiments, similar preincubation with other A₃ receptor antagonists was carried out. Preincubation with 8-*p*-sulfophenyltheophylline (10 μM), CGS 15943 (1 μM) (16), and MRS 1067 (1 μM) (12) failed to affect the level of subsequent A₃ receptor radioligand binding. Preincubation with compound **1b**, or its highly potent 4-nitrobenzyl derivative (12), at 100 nM did not significantly affect the level of subsequent A₃ receptor radioligand binding (loss of <10% of total binding). Compound **13**, another fluorosulfonyl derivative which bound considerably more weakly to human A₃ receptors, resulted in only 11 and 62% irreversible inhibition of radioligand binding at 1 and 5 μM, respectively.

DISCUSSION

Previously, we have applied a “functionalized congener approach” (21) to the design of ligands for G protein-coupled receptors, such as adenosine receptors (12), muscarinic receptors (35), and nucleotide receptors (36). By this approach, an easily derivatized functional group is incorporated at the end of a strategically designed and attached chain-substituent. Several of the derivatives in Table 1, such as the amines, **16** and **22**, and the carboxylic acids, **17** and **23**, represent easily reacted functionalized congeners of dihydropyridines, derivatives of which would retain moderate affinity and/or selectivity as A₃ receptor antagonists.

The indication that the 5-ester position is the most flexible for substitution in relation to A₃ receptor affinity has suggested the design of the amine-functionalized congener, **22**. As in

our previous studies of purines derivatized with long chains for the purpose of conjugating with other molecules while retaining the biological potency, this derivative may prove to be a key intermediate in the design of much higher molecular weight derivatives bearing “carrier” moieties, reporter groups, prosthetic groups, etc. The presence of the amino group in **22** also increased water solubility; unfortunately, it was not potent in binding at human A₃ receptors.

Irreversible inhibitors of binding of A₁ and A_{2A} subtypes of adenosine receptors have been reported (26–29). Furthermore, an irreversibly binding A₃ receptor agonist, MRS 1163, derived from IB-MECA was characterized (30). Among the pharmacological applications of such affinity labels are establishing the levels of spare receptors in native tissues, such as the heart (31–33). The irreversibly binding A₃ receptor antagonists presented in this study may prove to be useful tools in the characterization of this receptor subtype. Since the DHP antagonists (12) are selective for the human A₃ receptor vs other species, the design of irreversible ligands for A₃ receptors in rat (14) and other species remains to be accomplished.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of key A₃ adenosine receptor selective antagonists reported previously.

Figure 2. Irreversible inhibition of radioligand ($[^{125}\text{I}]\text{ABMECA}$) binding to human A_3 adenosine receptors in HEK-293 cell membranes, following a 1 h incubation with the sulfonyl fluoride derivative, **19** (n 2–6).

Scheme 1. Synthesis of 6-Phenyl-4-phenylethynyl-1,4-dihydropyridine Derivatives Using the Hantzsch Reaction^a

^aSilyl-, hydroxyl-, and thiol-containing ester groups are included. The 2-position contains either a methyl or a phenyl group.

Scheme 2.
Synthesis of β -Ketoesters

Scheme 3. Synthesis of Sulfonyl-, Carboxy-, and Amine-Functionalized Congeners of the A₃ Adenosine Receptor Selective Antagonists Based on Dihydropyridines^a

^a Reagents: (a) NBS; (b) CH₃OCH₂Cl, Et₃N; (c) K₂CO₃, acetone, reflux; (d) 1 N HCl, acetone, 45 °C; (e) H₂N(CH₂)₂NH₂, rt., 4 h; (f) N-hydroxysuccinimidy biotin, DMF, rt.

Scheme 4.

Synthesis of a 3-Carboxylic Acid Dihydropyridine Intermediate for Alkylation, as Shown in Scheme 3

Table 1

Affinities of 1,4-Dihydropyridine Derivatives in Radioligand Binding Assays at A₁, A_{2A}, and A₃ Receptors^{a-c}

compd	R ₃	R ₄	R ₅	K _i (μM) or % inhibition				
				rA ₁ ^a	rA _{2A} ^b	hA ₃ ^c	rA ₁ /hA ₃	
1b ^e	OCH ₂ CH ₃	Ph-C≡C-	OCH ₂ Ph	40.1 ± 7.5	d (10 ⁻⁴)	0.0314 ± 0.0028	1300	
2 ^e	OCH ₂ CH ₃	Ph-CH=CH- (trans)	OCH ₂ CH ₃	5.93 ± 0.27	4.77 ± 0.29	0.108 ± 0.012	55	
3 ^e	OCH ₂ CH ₃	Ph-C≡C-	OCH ₂ CH ₃	11.0 ± 0.1	26 ± 12% (10 ⁻⁴)	0.0766 ± 0.0151	140	
4 ^e	OCH ₂ Ph	Ph-C≡C-	OCH ₂ CH ₃	24 ± 4% (10 ⁻⁴)	d (10 ⁻⁴)	0.169 ± 0.026	>600	
5	OCH ₂ Ph	Ph-C≡C-	OCH ₂ Ph	d (10 ⁻⁴)	d (10 ⁻⁴)	0.195 ± 0.038	>500	
6, 2-Ph	OCH ₂ CH ₃	Ph-C≡C-	OCH ₂ Ph	36 ± 11% (10 ⁻⁴)	d (10 ⁻⁴)	20% (10 ⁻⁵)		
7, 2-Ph	OCH ₂ Ph	Ph-C≡C-	OCH ₂ Ph	19 ± 8% (10 ⁻⁴)	d (10 ⁻⁴)	d (10 ⁻⁵)		
8a	O(CH ₂) ₂ OCH ₃	Ph-C≡C-	OCH ₂ Ph	48.7 ± 7.3	14 ± 7% (10 ⁻⁴)	0.00191 ± 0.00031	25 000	
8b	O(CH ₂) ₂ OCH ₃	Ph-C≡C-	OCH ₂ Ph-4-NO ₂	23.6 ± 9.1	d (10 ⁻⁴)	0.00308 ± 0.00122	7700	
9	O(CH ₂) ₂ OH	Ph-C≡C-	OCH ₂ Ph	10.6 ± 1.9	d (10 ⁻⁴)	0.00207 ± 0.0012	5100	
10	O(CH ₂) ₂ SH	Ph-C≡C-	OCH ₂ Ph	d (10 ⁻⁴)	d (10 ⁻⁴)	0.197 ± 0.076	>500	
11	S(CH ₂) ₂ SH	Ph-C≡C-	OCH ₂ Ph	38 ± 11% (10 ⁻⁴)	d (10 ⁻⁴)	0.178 ± 0.018	>500	
12	O(CH ₂) ₂ S(CH ₂) ₂ Si(CH ₃) ₃	Ph-C≡C-	OCH ₂ Ph	13 ± 7% (10 ⁻⁴)	30% (10 ⁻⁴)	1.98 ± 0.64	>50	
13	OCH ₂ (4-SO ₂ F)Ph	Ph-C≡C-	OCH ₂ CH ₃	d (10 ⁻⁴)	18	0.341 ± 0.091	>300	
14	OCH ₂ (4-SO ₂ NH ₂)Ph	Ph-C≡C-	OCH ₂ CH ₃	16.9 ± 5.0	13.5 ± 1.3	0.187 ± 0.033	90	
15	OCH ₂ (4-CO ₂ CH ₂ CCl ₃)Ph	Ph-C≡C-	OCH ₂ CH ₃	19% (10 ⁻⁴)	5.99 ± 1.67	8.93 ± 5.63	>1	
16	OCH ₂ Ph-4-CO-NH(CH ₂) ₂ NH ₂	Ph-C≡C-	OCH ₂ CH ₃	6.65 ± 1.35	7.85	0.128 ± 0.018	52	
17	OCH ₂ (4-CO ₂ H)Ph	Ph-C≡C-	OCH ₂ CH ₃	16.0 ± 2.1	<1 (shallow curves)	0.799 ± 0.297	20	
18	OCH ₂ Ph-4-CO-NH(CH ₂) ₂ NH-biotin	Ph-C≡C-	OCH ₂ CH ₃	38 ± 2% (10 ⁻⁴)	16 ± 8% (10 ⁻⁴)	0.599 ± 0.151	>100	
19 ^f	OCH ₂ CH ₃	Ph-C≡C-	OCH ₂ (4-SO ₂ F)Ph	41 ± 3% (10 ⁻⁴)	20% (10 ⁻⁴)	0.00242 ± 0.00032	>30 000	
20	OCH ₂ CH ₃	Ph-C≡C-	OCH ₂ (4-SO ₂ NH ₂)Ph	14.4 ± 2.4	22.0 ± 6.1	0.292 ± 0.030	49	
21	OCH ₂ CH ₃	Ph-C≡C-	OCH ₂ (4-CO ₂ CH ₂ CCl ₃)Ph	33 ± 6% (10 ⁻⁴)	11% (10 ⁻⁴)	5.00	>20	
22 ^g	OCH ₂ CH ₃	Ph-C≡C-	OCH ₂ Ph-4-CO-NH(CH ₂) ₂ NH ₂	3.0	39 ± 4% (10 ⁻⁴)	0.697 ± 0.259	4.3	

compd	R ₃	R ₄	R ₅	K _i (μM) or % inhibition			
				rA ₁ ^a	rA _{2A} ^b	hA ₃ ^c	rA ₁ /hA ₃
23	OCH ₂ CH ₃	Ph-C≡C-	OCH ₂ (4-CO ₂ H)Ph	19.0 ± 5.3	8.22 ± 3.76	d (10 ⁻⁶)	

^aDisplacement of specific [³H]R-PIA binding in rat brain membranes, expressed as K_i ± SEM (*n* = 2–5), or as a percentage of specific binding displaced at the indicated concentration (M).

^bDisplacement of specific [³H]CGS 21 680 binding in rat striatal membranes, expressed as K_i ± SEM (*n* = 2–6), or as a percentage of specific binding displaced at the indicated concentration (M).

^cDisplacement of specific [¹²⁵I]AB-MECA binding at human A₃ receptors expressed in HEK cells, in membranes, expressed as K_i ± SEM (*n* = 2–4).

^dDisplacement of 10% of specific binding at the indicated concentration (M).

^eValues taken from Jiang et al. (12) and references therein.

^fBound to human A₃ receptors irreversibly (see Figure 2). K_i value is apparent.

^gSame as compound 25 in Jiang et al. (12) K_i values have been redetermined.

Table 2

Characterization of Dihydropyridine Derivatives

no	formula	MS	analysis	yield (%)
5	C ₃₆ H ₂₉ NO ₄	539 (EI)	HRMS	11
6	C ₃₆ H ₂₉ NO ₄	539 (EI)	HRMS	40
7	C ₄₁ H ₃₁ NO ₄ ·0.5H ₂ O	601 (EI)	C, H, N	50
8a	C ₃₂ H ₂₉ NO ₅	507 (EI)	HRMS	15
8b	C ₃₂ H ₂₈ N ₂ O ₇	570 (CI, M ⁺ + NH ₄)	C, H, N	23
9	C ₃₁ H ₂₇ NO ₅	493 (EI)	C, H, N	72
10	C ₃₁ H ₂₇ NO ₄ S	509 (EI)	C, H, N	22
11	C ₃₁ H ₂₇ NO ₃ S ₂ ·1.0H ₂ O	525 (EI)	C, H, N	16
12	C ₃₆ H ₃₉ NO ₄ SSi	609 (CI)	HRMS	28
13	C ₃₁ H ₂₆ NFO ₆ S·0.71EtOAc	559 (EI)	C, H, N	78
14	C ₃₁ H ₂₈ N ₂ O ₆ S	556 (EI)	HRMS(FAB)	49
15	C ₃₄ H ₂₈ NCl ₃ O ₆	652 (CI)	C, H, N	89
16	C ₃₄ H ₃₃ N ₃ O ₅	564 (FAB)	HRMS(FAB)	59
17	C ₃₂ H ₂₇ NO ₆	521 (EI)	HRMS(FAB)	38
18	C ₄₄ H ₄₇ N ₅ O ₇ S	790 (CI)	HRMS(FAB)	64
19	C ₃₁ H ₂₆ NFO ₆ S	559 (EI)	C, H, N	75
20	C ₃₁ H ₂₈ N ₂ O ₆ S	556 (EI)	HRMS(FAB)	56
23	C ₃₂ H ₂₇ NO ₆ ·0.92H ₂ O	521 (EI)	C, H, N	51