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Click Modification in the N^6 Region of A_3 Adenosine Receptor-Selective Carbocyclic Nucleosides for Dendrimeric Tethering that Preserves Pharmacophore Recognition

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Abstract

Adenosine derivatives were modified with alkynyl groups on N^6 substituents for linkage to carriers using Cu(I)-catalyzed click chemistry. Two parallel series, both containing a rigid North-methanocarba (bicyclo[3.1.0]hexane) ring system in place of ribose, behaved as A_3 adenosine receptor (AR) agonists: (5'-methyluronamides) or partial agonists (4'-truncated). Terminal alkynyl groups on a chain at the 3 position of a N^6 -benzyl group or simply through a N^6 -propargyl group were coupled to azido derivatives, which included both small molecules and G4 (fourth-generation) multivalent poly(amidoamine) (PAMAM) dendrimers, to form 1,2,3-triazolyl linkers. The small molecular triazoles probed the tolerance in A_3 AR binding of distal, sterically bulky groups such as 1-adamantyl. Terminal 4-fluoro-3-nitrophenyl groups anticipated nucleophilic substitution for chain extension and ^{18}F radiolabeling. N^6 -(4-Fluoro-3-nitrophenyl)-triazolylmethyl derivative **32** displayed a K_i of 9.1 nM at A_3 AR with ~1000-fold subtype selectivity. Multivalent conjugates additionally containing click-linked water-solubilizing polyethylene glycol groups potently activated A_3 AR in the 5'-methyluronamide, but not 4' truncated series. N^6 -Benzyl nucleoside conjugate **43** (apparent K_i 24 nM) maintained binding affinity of the monomer better than a N^6 -triazolylmethyl derivative. Thus, the N^6 region of 5'-methyluronamide derivatives, as modeled in receptor docking, is suitable for functionalization and tethering by click chemistry to achieve high A_3 AR agonist affinity and enhanced selectivity.

Keywords

G protein-coupled receptor; PAMAM dendrimer; purines; structure activity relationship; molecular modeling; adenylate cyclase

INTRODUCTION

Selective ligands of the A_3 adenosine receptor (AR), a G protein-coupled receptor (GPCR), have antiinflammatory, anticancer, antiischemic, and myeloprotective activities.^{1,2} A_3 AR agonists are already in clinical trials for liver cancer, rheumatoid arthritis, psoriasis, and dry eye disease, with additional envisioned applications for bowel inflammation, ischemia, and

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Supporting Information Available: ^1H NMR and mass spectra of representative compounds and modeling procedures. This material is available free of charge via the Internet at <http://pubs.acs.org/BC>.

other autoimmune inflammatory disorders.³⁻⁷ The feasibility of using A₃AR antagonists for the treatment of glaucoma has been demonstrated in animal models.⁸⁻¹⁰

Poly(amidoamine) (PAMAM) dendrimers may serve as biocompatible carriers for various GPCR drugs, for example, multivalent nucleoside conjugates for activation of ARs that do not require drug cleavage.¹¹⁻¹³ Such GPCR ligand-dendrimer (GLiDe) conjugates are sufficiently large to bridge multiple binding sites present in GPCR dimers and other higher-order receptor aggregates and have already demonstrated enhanced pharmacological properties in comparison to the monomeric ligands.¹⁴⁻¹⁸

Monomeric nucleoside derivatives that bind selectively to the A₃AR have demonstrated a range of efficacies, from antagonist to full agonist,¹⁹ and detailed structure activity relationship (SAR) studies have already been conducted. A prototypical A₃AR agonist, e.g. ribose 5'-*N*-methylonamide Cl-IB-MECA **1**, is highly receptor subtype-selective and has entered clinical trials for liver cancer.³ The 4'-thio modification, present in the potent agonist **2**, was found to be well-tolerated in receptor binding.²⁰ Later generation carbocyclic nucleosides containing a North (N)-methanocarba (bicyclo[3.1.0]hexane) ring system in place of the ribose tetrahydrofuryl moiety have led to A₃AR agonists of high affinity and selectivity. These agonists include the anti-ischemic nucleoside MRS3558 **3** and its 3-iodo analogue **4**, in which the rigid, fused bicyclic system maintains a receptor-preferred conformation.²¹ A parallel approach to selective binding to the A₃AR is based on truncation of the 5'-uronamide group in ribonucleosides, 4'-thionucleosides, and carbocyclics, as in **5** – **8**. The effect of truncation is to reduce efficacy in activation of the receptor, while not precluding selectivity of binding to the A₃AR. Thus, truncated oxo **5** and thio **6** nucleosides are antagonists of the A₃AR, and truncated 3-halo substituted **7** and **8** are partial agonists.²²⁻²⁴ Compound **6** lowered intraocular pressure in a mouse model of glaucoma,⁹ an action characteristic of A₃AR antagonists, and compounds **7** and **8** were radiolabeled with ⁷⁶Br and ¹²⁵I, respectively, for use as radioligand probes of the A₃AR in vitro and in vivo.²⁵ These agonists, antagonists, and partial agonists all contained a 3-substituted benzyl group at the N⁶ position, which enhanced A₃AR selectivity.

This study applies to multivalent GLiDe conjugates the SAR developed for monomeric nucleosides as receptor agonists. The linking step involved click chemistry, which encompasses various principles of reactivity and has been used extensively for orthogonal coupling of small molecules to polymers.^{26,27} In particular, Cu(I)-catalyzed [3 + 2] cycloaddition reactions that combine azido and acetylene moieties to form triazole rings are a convenient and efficient means of linking two molecules.²⁸ Recently, we incorporated dialkynyl groups on an extended adenine C2 substituent of AR agonists for tethering to PAMAM dendrimers by Cu(I)-catalyzed click chemistry.²⁹ In the present series, we have applied this chemistry as a functionalization approach around the N⁶ region of adenosine and its family of A₃AR agonists. As with the C2-conjugated series, the proximal alkyne is designed to promote receptor recognition, and the distal alkyne may serve as a site for coupling to macromolecular carriers while maintaining receptor recognition. Thus, we have used click chemistry to identify another region on the nucleoside for covalent attachment that is distal from the pharmacophore and does not interfere with binding/activation. This region of the nucleosides is predicted by modeling to extend into the extracellular portion of the A₃AR.^{23,30}

EXPERIMENTAL PROCEDURES

Chemistry

General Methods—All reagents and solvents (regular and anhydrous) were of analytical grade and obtained from commercial suppliers and used without further purification.

Reactions were conducted under an atmosphere of nitrogen whenever anhydrous solvents were used. All reactions were monitored by thin-layer chromatography (TLC) using silica gel coated plates with a fluorescence indicator which were visualized: (a) under UV light, (b) by dipping in a mixture of anisaldehyde (2.5 mL)/ conc. H₂SO₄ (5 mL)/methanol (425 mL) or (c) by dipping the plate in a solution of ninhydrin (0.3 g in 100 mL EtOH, containing AcOH, 1.3 mL) followed by heating. Silica gel column chromatography was performed with silica gel (SiO₂, 200-400 mesh, 60 Å) using moderate air pressure. Evaporation of solvents was carried out under reduced pressure at a temperature below 50 °C. After column chromatography, appropriate fractions were pooled, evaporated, and dried at high vacuum for at least 12 h to give the desired products in high purity. ¹H NMR ascertained sample purity and spectra were recorded with a Bruker 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane or using deuterated solvent as the internal standard (δH: CDCl₃ 7.26 ppm). For ¹⁹F NMR spectra, CF₃CO₂H was set as 0 ppm. ESI - High Resolution Mass Spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine. Observed mass accuracies are those expected on the basis of known performance of the instrument as well as the trends in masses of standard compounds observed at intervals during the series of measurements. Reported masses are observed masses uncorrected for this time-dependent drift in mass accuracy.

(1S, 2R, 3S, 4R, 5S)-4-[2-Chloro-6-{3-(1,6-heptadiynyl)}-9H-purin -9-yl]- 2, 3-dihydroxybicyclo[3.1.0]hexane-1-carboxylic acid N-methylamide (9)—To a solution of compound **4** (50 mg, 0.09 mmol) in anhydrous DMF (2 mL), PdCl₂(PPh₃)₂ (12.6 mg, 0.01 mmol), CuI (1.7 mg, 0.008 mmol), 1,6-heptadiyne (0.1 mL, 0.9 mmol) and then triethylamine (0.12 mL, 0.89 mmol) were added and stirred overnight at room temperature. Solvent was evaporated under vacuum, and the residue was purified on flash silica gel column chromatography (CH₂Cl₂:MeOH = 60:1) to give compound **9** (38 mg, 81%) as syrup. ¹H NMR (CDCl₃, 400 MHz) δ 7.8 (s, 1H), 7.42 (s, 1H), 7.24-7.30 (m, 3H), 5.03 (d, *J* = 6.0 Hz, 1H), 4.84 (s, 1H), 4.79 (br s, 1H), 4.13 (d, *J* = 6.4 Hz, 1H), 2.94 (s, 3H), 2.55 (t, *J* = 7.2 Hz, 2H), 2.36-2.41 (m, 2H), 2.18-2.22 (m, 1H), 2.00 (t, *J* = 2.8 Hz, 1H), 1.68 (t, *J* = 9.2 Hz, 1H), 1.85-1.79 (m, 2H), 1.37-1.41 (m, 1H). HRMS calculated for C₂₇H₂₈ClN₆O₃ (M + H)⁺: 519.1911; found 519.1930.

(1S, 2R, 3S, 4R, 5S)-4-[2-Chloro-6-{3-(1,7-octadiynyl)}-9H-purin -9-yl]- 2, 3-dihydroxybicyclo[3.1.0]hexane-1-carboxylic acid N-methylamide (10)—Compound **10** (85%) was synthesized from compound **4** and 1,7-octadiyne following the same procedure as for compound **2**. ¹H NMR (CD₃OD, 400 MHz) δ 8.04 (s, 1H), 7.40 (s, 1H), 7.26-7.34 (m, 3H), 5.08 (d, *J* = 6.8 Hz, 1H), 4.81 (s, 1H), 4.73 (br s, 2H), 4.60 (br s, 2H), 4.01 (d, *J* = 6.4 Hz, 1H), 2.87 (s, 3H), 2.43 (t, *J* = 6.8 Hz, 2H), 2.21-2.26 (m, 2H), 2.05-2.08 (m, 1H), 1.68-1.71 (m, 1H), 1.38-1.40 (m, 1H). HRMS calculated for C₂₈H₃₀ClN₆O₃ (M + H)⁺: 533.2068; found 533.2070.

(1S, 2R, 3S, 4R, 5S)-4-[2-Chloro-6-{3-(1,8-nonadiynyl)}-9H-purin -9-yl]- 2, 3-dihydroxybicyclo[3.1.0]hexane-1-carboxylic acid N-methylamide (11)—Compound **11** (79%) was synthesized from compound **4** and 1,8-nonadiyne following the same procedure as for compound **2**. ¹H NMR (CDCl₃, 400 MHz) δ 7.92 (s, 1H), 7.43 (s, 1H), 7.25-7.32 (m, 3H), 5.10 (d, *J* = 6.6 Hz, 1H), 4.85 (s, 1H), 4.78 (br s, 2H), 4.15 (d, *J* = 6.3 Hz, 1H), 2.93 (s, 3H), 2.42 (t, *J* = 6.0 Hz, 2H), 2.22-2.23 (m, 2H), 1.97 (t, *J* = 2.8 Hz, 1H), 1.71-1.78 (m, 2H), 1.58-1.61 (m, 6H), 1.48-1.52 (m, 1H). HRMS calculated for C₂₉H₃₂ClN₆O₃ (M + H)⁺: 547.2224; found 547.2239.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(5-(1-(4-fluoro-3-nitrophenyl)-1H-1,2,3-triazol-4-yl)pent-1-ynyl)benzylamino)-9H-purin-9-yl)-2,3-dihydroxybicyclo[3.1.0]hexane-1-carboxylic acid-N-methylamide (12)—4-

Fluoro-3-nitro-phenylazide (9.3 mg, 0.051 mmol) and TBTA³¹ (1 mg, 0.001 mmol) were added to a solution of compound **9** (19 mg, 0.036 mmol) in *t*-butanol (0.6 mL)-CH₂Cl₂ (0.6 mL)-water (0.6 mL) at room temperature. Freshly prepared 1M solution of sodium ascorbate (36.6 μL) followed by 7.5% solution of copper sulfate (60.7 μL, 0.018 mmol) was also added into the reaction mixture and stirred at room temperature overnight. Solvent was evaporated, and the residue was purified on flash silica gel column chromatography (CH₂Cl₂:MeOH = 40:1) to give compound **12** (23 mg, 90%) as a syrup. ¹H NMR (CD₃OD, 400 MHz) δ 8.53- 8.62 (m, 1H), 8.49 (s, 1H), 8.22-8.19 (m, 1H), 8.04 (s, 1H), 7.60-7.67 (m, 2H), 7.39-23 (m, 3H), 5.05 (d, *J* = 5.6 Hz, 1H), 4.81 (s, 1H), 4.71 (br s, 2H), 4.00 (d, *J* = 6.8 Hz, 1H), 2.98 (t, *J* = 7.6 Hz, 2H), 2.86 (s, 3H), 2.53 (t, *J* = 6.8 Hz, 2H), 2.02-2.08 (m, 3H), 1.82 (t, *J* = 4.8 Hz, 1H), 1.36-1.39 (m, 1H). HRMS calculated for C₃₃H₃₁ClFN₈O₃ (M + H)⁺: 701.2151; found 701.2175.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(6-(1-(4-fluoro-3-nitrophenyl)-1H-1,2,3-triazol-4-yl)hex-1-ynyl)benzylamino)-9H-purin-9-yl)-2,3-dihydroxybicyclo[3.1.0]hexane-1-carboxylic acid-N-methylamide (13)—

Compound **13** (94%) was synthesized from compound **10** following the same procedure as for compound **12**. ¹H NMR (CD₃OD, 400 MHz) δ 8.65-8.58 (m, 1H), 8.45 (s, 1H), 8.23-8.19 (m, 1H), 8.03 (s, 1H), 7.65 (t, *J* = 8.8 Hz, 1H), 7.41 (s, 1H), 7.33-7.25 (m, 2H), 5.07 (d, *J* = 6.8 Hz, 1H), 4.80 (s, 1H), 4.72 (br s, 2H), 4.00 (d, *J* = 6.8 Hz, 1H), 2.87 (s, 3H), 2.51 (t, *J* = 6.8 Hz, 2H), 2.07-2.04 (m, 1H), 1.97-1.91 (m, 2H), 1.82 (t, *J* = 5.2 Hz, 2H), 1.74-1.69 (m, 2H), 1.39-1.35 (m, 1H). HRMS calculated for C₃₄H₃₃ClFN₁₀O₅ (M + H)⁺: 715.2308; found 715.2302.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(7-(1-(4-fluoro-3-nitrophenyl)-1H-1,2,3-triazol-4-yl)hept-1-ynyl)benzylamino)-9H-purin-9-yl)-2,3-dihydroxybicyclo[3.1.0]hexane-1-carboxylic acid-N-methylamide (14)—

Compound **14** (96%) was synthesized from compound **11** following the same procedure as for compound **12**. ¹H NMR (CD₃OD, 400 MHz) δ 8.55-8.59 (m, 1H), 8.41 (s, 1H), 8.16-8.12 (m, 1H), 8.03 (s, 1H), 7.60 (t, *J* = 9.2 Hz, 1H), 7.36 (s, 1H), 7.30-7.20 (m, 2H), 5.08 (d, *J* = 6.4 Hz, 1H), 4.80 (s, 1H), 4.69 (br s, 2H), 4.01 (d, *J* = 6.4 Hz, 1H), 2.87-2.82 (m, 5H), 2.44 (t, *J* = 6.4 Hz, 2H), 2.08-2.05 (m, 1H), 1.84-1.81 (m, 3H), 1.68-1.58 (m, 4H), 1.39-1.38 (m, 1H). HRMS calculated for C₃₅H₃₅ClFN₁₀O₅ (M + H)⁺: 729.2464; found 729.2463.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(6-(1-(adamantyl)-1H-1,2,3-triazol-4-yl)hex-1-ynyl)benzylamino)-9H-purin-9-yl)-2,3-dihydroxybicyclo[3.1.0]hexane-1-carboxylic acid-N-methylamide (15)—

Compound **15** (93%) was synthesized from compound **10** following the same procedure as for compound **12**. ¹H NMR (CD₃OD, 400 MHz) δ 8.04 (s, 1H), 7.40 (s, 1H), 7.35-7.26 (m, 3H), 5.09 (d, *J* = 6.8 Hz, 1H), 4.82 (s, 1H), 4.73 (s, 2H), 4.02 (d, *J* = 6.8 Hz, 1H), 2.87 (s, 1H), 2.44 (t, *J* = 6.8 Hz, 2H), 2.26-2.21 (m, 3H), 2.17 (m, 8H), 2.08-2.05 (m, 1H), 1.82 (t, *J* = 4.8 Hz, 1H), 1.70-1.69 (m, 10H), 1.39-1.36 (m, 1H), 0.94-0.88 (m, 1H). HRMS calculated for C₃₈H₄₅ClN₉O₃ (M + H)⁺: 710.3257; found 710.3248.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(5-(1-(4-fluoro-3-amino-phenyl)-1H-1,2,3-triazol-4-yl)pent-1-ynyl)benzylamino)-9H-purin-9-yl)-2,3-dihydroxybicyclo[3.1.0]hexane-1-carboxylic acid-N-methylamide (16)—Zinc

dust (3 mg, 0.045 mmol) was added to a solution of compound **12** (5 mg, 0.007 mmol) in

acetonitrile (0.5 mL)-acetic acid (0.5 mL) at 0 °C. After addition, the reaction mixture was brought to room temperature and stirred for 2 h under the same conditions. The reaction mixture was filtered on a Celite bed, and the filtrate was evaporated under vacuum. The residue was purified on flash silica gel column chromatography (CH₂Cl₂ : MeOH = 20:1) to give compound **16** (3 mg, 62%). ¹H NMR (CD₃OD, 400 MHz) δ 8.65-8.58 (m, 1H),

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(7-(1-(4-fluoro-3-amino-phenyl)-1H-1,2,3-triazol-4-yl)hept-1-ynyl)benzylamino)-9H-purin-9-yl)-2,3-dihydroxybicyclo[3.1.0]hexane-1-carboxylic acid-N-methylamide (17)—

Compound **17** (64%) was synthesized from compound **14** following the same procedure as for compound **16**. ¹H NMR (CD₃OD, 400 MHz) δ 8.12 (s, 1H), 8.03 (s, 1H), 7.38 (s, 1H), 7.32-7.21 (m, 4H), 7.06-7.02 (m, 1H), 6.90-6.86 (m, 1H), 5.08 (d, *J* = 7.2 Hz, 1H), 4.80 (s, 1H), 4.70 (br s, 2H), 4.01 (d, *J* = 6.4 Hz, 1H), 2.87 (s, 3H), 2.80 (t, *J* = 7.2 Hz, 2H), 2.44 (t, *J* = 6.8 Hz, 2H), 2.02-2.09 (m, 1H), 1.83-1.78 (m, 2H), 1.68-1.54 (m, 5H), 0.89-0.93 (m, 1H). HRMS calculated for C₄₀H₃₇ClFN₈O (M + H)⁺: 699.2763; found 699.2745.

(1R, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(hepta-1,6-diynyl)benzylamino)-9H-purin-9-yl) 2,3-O-isopropylidene-bicyclo[3.1.0]hexane (19)—PdCl₂(PPh₃)₂ (29.7 mg, 0.04 mmol), CuI (8.0 mg, 0.04 mmol), and 1,6-heptadiyne (0.24 mL, 2.11 mmol)

followed by triethylamine (60 μL, 0.41 mmol) were added to a solution of compound **18** (114 mg, 0.211 mmol) in anhydrous DMF (2 mL), and the reaction mixture was stirred overnight at room temperature. Solvent was evaporated under vacuum, and the residue was purified on flash silica gel column chromatography (hexane:ethyl acetate = 2:1) to give compound **19** (83 mg, 78%) as foamy syrup. ¹H NMR (CDCl₃, 400 MHz) δ 7.72 (s, 1H), 7.42 (s, 1H), 7.28-7.42 (m, 3H), 6.31 (br s, 1H), 5.35 (t, *J* = 6.0 Hz, 1H), 4.98 (s, 1H), 4.81 (br s, 2H), 4.65 (d, *J* = 7.6 Hz, 1H), 2.55 (t, *J* = 7.2 Hz, 2H), 2.38 (dd, *J*₁ = 2.8 Hz, *J*₂ = 3.2 Hz, 2H), 2.08-2.14 (m, 1H), 1.99 (t, *J* = 2.8 Hz, 1H), 1.82-1.85 (m, 2H), 1.64-1.67 (m, 2H), 1.56 (s, 3H), 1.26 (s, 3H), 0.93-1.00 (m, 1H). HRMS calculated for C₂₈H₂₉ClN₅O₂ (M + H)⁺: 502.2032; found 502.2029.

(1R, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(nona-1,8-diynyl)benzylamino)-9H-purin-9-yl) 2,3-O-isopropylidene-bicyclo[3.1.0]hexane (21)—Compound **21** (80%)

was synthesized from compound **18** and 1,8-nonadiyne following the same procedure as for compound **19**. ¹H NMR (CDCl₃, 400 MHz) δ 7.71 (s, 1H), 7.42 (s, 1H), 7.27-7.34 (m, 3H), 6.33 (br s, 1H), 5.35 (t, *J* = 6.4 Hz, 1H), 4.98 (s, 1H), 4.80 (br s, 2H), 4.64 (d, *J* = 7.2 Hz, 1H), 2.41 (t, *J* = 3.6 Hz, 2H), 2.28-2.21 (m, 3H), 2.14-2.08 (m, 1H), 1.97-1.96 (t, *J* = 2.8 Hz, 1H), 1.54-1.68 (m, 10H), 1.26 (s, 3H), 0.88-0.99 (m, 1H). HRMS calculated for C₃₀H₃₃ClN₅O₂ (M + H)⁺: 530.2323; found 530.2315.

(1R, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(hepta-1,6-diynyl)benzylamino)-9H-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (22)—A solution of compound **19** (92 mg,

0.18 mmol) in CH₂Cl₂ (4 mL) and trifluoromethane sulfonic acid:water (2:1, 3 mL) was stirred at room temperature overnight. Solvent was evaporated, and the residue was purified on flash silica gel chromatography (CH₂Cl₂:MeOH = 70:1) to give compound **22** (77 mg, 92%) as a syrup. ¹H NMR (CDCl₃, 400 MHz) δ 7.80 (s, 1H), 7.42 (s, 1H), 7.29-7.35 (m, 3H), 6.41 (br s, 1H), 4.87 (s, 1H), 4.81 (br s, 2H), 4.02 (d, *J* = 6.4 Hz, 1H), 4.00 (s, 1H), 2.69 (d, *J* = 6.3 Hz, 1H), 2.56 (t, *J* = 6.8 Hz, 2H), 2.36-2.41 (m, 2H), 2.03-2.08 (m, 1H), 2.00 (t, *J* = 2.8 Hz, 1H), 1.85-1.82 (m, 2H), 1.68-1.64 (m, 1H), 1.26-1.30 (m, 2H), 0.90-0.80 (m, 1H). HRMS calculated for C₂₅H₂₅ClN₅O₂ (M + H)⁺: 462.1697; found 462.1696.

(1R, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(octa-1,7-diynyl)benzylamino)-9H-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (23)—PdCl₂(PPh₃)₂ (5.7 mg, 0.008 mmol), CuI (1.0

mg, 0.005 mmol), and 1,7-octadiyne (27 μ L, 0.2 mmol) followed by triethylamine (57 μ L, 0.4 mmol) were added to a solution of compound **18** (20.4 mg, 0.04 mmol) in anhydrous DMF (1.2 mL) and the reaction mixture was stirred overnight at room temperature. Solvent was evaporated under vacuum, and the residue was roughly purified on flash silica gel column chromatography and the obtained product was dissolved in CH_2Cl_2 (3 mL) and 2 mL of trifluoromethane sulfonic acid-water (2:1) were added into it and stirred at room temperature overnight. Solvent was evaporated, and the residue was purified on flash silica gel chromatography (CH_2Cl_2 :MeOH = 60:1) to give compound **23** (12.6 mg, 70%) as a syrup. ^1H NMR (CDCl_3 , 400 MHz) δ 7.80 (s, 1H), 7.42 (s, 1H), 7.35-7.29 (m, 3H), 6.43 (br s, 1H), 4.88 (s, 1H), 4.80 (br s, 2H), 4.03 (d, J = 7.2 Hz, 1H), 3.97 (s, 1H), 2.64 (m, 1H), 2.45 (t, J = 6.4 Hz, 2H), 2.29-2.26 (m, 3H), 2.08-2.06 (m, 1H), 1.98 (t, J = 2.4 Hz, 1H), 1.71-1.66 (m, 3H), 1.28-1.31 (m, 1H), 0.86-0.82 (m, 1H). HRMS calculated for $\text{C}_{26}\text{H}_{27}\text{ClN}_5\text{O}_2$ ($M + \text{H}$) $^+$: 476.1853; found 476.1869.

(1R, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(nona-1,8-diynyl)benzylamino)-9H-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (24)—Compound **24** (89%) was synthesized from compound **21** following the same procedure as for compound **22**. ^1H NMR (CD_3OD , 400 MHz) δ 8.15 (s, 1H), 7.40 (s, 1H), 7.26-7.33 (m, 3H), 4.80 (s, 1H), 4.74-4.69 (m, 1H), 4.60 (br s, 2H), 3.92 (d, J = 6.4 Hz, 1H), 2.41 (t, J = 6.4 Hz, 2H), 2.18-2.19 (m, 3H), 2.01-1.96 (m, 1H), 1.70-1.65 (m, 1H), 1.59-1.50 (m, 6H), 1.34-1.30 (m, 1H), 0.80-0.74 (m, 1H). HRMS calculated for $\text{C}_{27}\text{H}_{29}\text{ClN}_5\text{O}_2$ ($M + \text{H}$) $^+$: 490.2010; found 490.2000.

(1R, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(5-(1-(4-fluoro-3-nitrophenyl)-1H-1,2,3-triazol-4-yl)pent-1-ynyl)benzylamino)-9H-purin-9-yl)-bicyclo[3.1.0]hexane-2,3-diol (25)—4-Fluoro-3-nitro-phenylazide (7.5 mg, 0.041 mmol) and TBTA (1 mg, 0.001 mmol) were added to a solution of compound **22** (13.5 mg, 0.029 mmol) in CH_2Cl_2 -*t*-butanol (0.6 mL)-water (0.6 mL) at room temperature. Freshly prepared 1M solution of sodium ascorbate (29.2 μ L) followed by 7.5% solution of copper sulfate (48 μ L, 0.015 mmol) was also added into the reaction mixture stirred at room temperature overnight. Solvent was evaporated, and the residue was purified on flash silica gel column chromatography (CH_2Cl_2 :MeOH = 45:1) to give compound **25** (17 mg, 91%) as a syrup. ^1H NMR (CD_3OD , 400 MHz) δ 8.61-8.59 (m, 1H), 8.49 (s, 1H), 8.23-8.19 (m, 1H), 8.14 (s, 1H), 7.65 (t, J = 9.2 Hz, 1H), 7.39 (s, 1H), 7.33-7.24 (m, 3H), 4.79 (s, 1H), 4.72-4.70 (m, 3H), 3.90 (d, J = 6.4 Hz, 1H), 2.99 (t, J = 7.6 Hz, 2H), 2.53 (t, J = 6.8 Hz, 2H), 2.08-1.96 (m, 3H), 1.69-1.65 (m, 1H), 1.33-1.30 (m, 1H), 0.79-0.75 (m, 1H). HRMS calculated for $\text{C}_{31}\text{H}_{28}\text{ClFN}_9\text{O}_4$ ($M + \text{H}$) $^+$: 644.1937; found 644.1962.

(1R, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(3-(6-(1-(4-fluoro-3-nitrophenyl)-1H-1,2,3-triazol-4-yl)hex-1-ynyl)benzylamino)-9H-purin-9-yl)-bicyclo[3.1.0]hexane-2,3-diol (26)—Compound **26** (95%) was synthesized from compound **23** following the same procedure as for compound **25**. ^1H NMR (CDCl_3 , 400 MHz) δ 8.44-8.39 (m, 1H), 8.07-8.11 (m, 1H), 7.83 (s, 1H), 7.77 (s, 1H), 7.51-7.43 (m, 2H), 7.33-7.26 (m, 3H), 6.58 (br s, 1H), 4.87 (s, 1H), 4.79 (br s, 2H), 4.15 (s, 1H), 4.02 (d, J = 6.8 Hz, 1H), 2.89 (t, J = 7.6 Hz, 2H), 2.49 (t, J = 6.8 Hz, 2H), 2.08-2.04 (m, 1H), 1.96-1.90 (m, 2H), 1.76-1.64 (m, 3H), 1.31-1.28 (m, 1H), 0.79-0.85 (m, 1H). HRMS calculated for $\text{C}_{32}\text{H}_{30}\text{ClFN}_9\text{O}_4$ ($M + \text{H}$) $^+$: 658.2093; found 658.2124.

(1R,2R,3S,4R,5S)-4-(2-Chloro-6-(3-(7-(1-(4-fluoro-3-nitrophenyl)-1H-1,2,3-triazol-4-yl)hept-1-ynyl)benzylamino)-9H-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (27)—Compound **27** (91%) was synthesized from compound **24** following the same procedure as for compound **25**. ^1H NMR (CD_3OD , 400 MHz) δ 8.56-8.54 (m, 1H), 8.40 (s, 1H), 8.13-8.18 (m, 2H), 7.60 (t, J = 8.8 Hz, 1H), 7.37 (s, 1H), 7.30 (d, J = 6.8 Hz, 1H),

7.24-7.21 (m, 2H), 4.79 (s, 1H), 4.72-4.69 (m, 3H), 3.90 (d, $J = 6.4$ Hz, 1H), 2.84 (t, $J = 7.2$ Hz, 2H), 2.45 (t, $J = 6.8$ Hz, 2H), 2.01-1.95 (m, 1H), 1.86-1.79 (m, 2H), 1.68-1.58 (m, 5H), 1.33-1.30 (m, 1H), 0.82-0.72 (m, 1H). HRMS calculated for $C_{33}H_{32}ClFN_9O_4$ ($M + H$)⁺: 672.2250; found 672.2245.

(1S, 2R, 3S, 4R, 5S)-Ethyl-4-(2-chloro-6-(prop-2-ynylamino)-9H-purin-9-yl)-2,3-O-isopropylidene-bicyclo[3.1.0]hexane-1-carboxylate (29)—Propargylamine (0.5 mL, 7.5 mmol) and triethylamine (2.1 mL, 15 mmol) were added to a solution of compound **28** (620 mg, 0.14 mmol) in anhydrous methanol (10 mL) and stirred overnight at room temperature. Solvent was evaporated, and the residue was purified on flash silica gel column chromatography (hexane:ethyl acetate=1:1) to give compound **29** (511 mg, 79%) as a syrup. ¹H NMR (CDCl₃, 400 MHz) δ 7.73 (s, 1H), 6.20 (br s, 1H), 5.89 (d, $J = 7.0$ Hz, 1H), 4.88 (s, 1H), 4.74 (d, $J = 7.1$ Hz, 1H), 4.47 (br s, 2H), 4.32-4.28 (m, 2H), 4.27-4.13 (m, 1H), 2.32 (s, 1H), 2.24-2.20 (m, 1H), 1.76-1.72 (m, 1H), 1.68 (s, 3H), 1.57-1.53 (m, 4H), 1.36 (t, $J = 7.2$ Hz, 3H), 1.30-1.25 (m, 1H). HRMS calculated for $C_{20}H_{23}ClN_5O_4$ ($M + H$)⁺: 432.1367; found 432.1362.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(prop-2-ynylamino)-9H-purin-9-yl)-2,3-O-isopropylidene-N-methyl bicyclo[3.1.0]hexane-1-carboxamide (30)—Methylamine (40% solution, 3 mL) was added to a solution of compound **29** (244 mg, 0.56 mmol) in methanol (9 mL) and stirred for 2 d at room temperature. Solvent was evaporated under vacuum, and the residue was purified on flash silica gel column chromatography (CH₂Cl₂:MeOH = 70:1) to give compound **30** (148 mg, 63%) as a syrup. ¹H NMR (CD₃OD, 400 MHz) δ 8.08 (s, 1H), 5.75 (d, $J = 7.2$ Hz, 1H), 4.97 (s, 1H), 4.84 (d, $J = 6.8$ Hz, 1H), 4.35 (br s, 2H), 2.86 (s, 3H), 2.63 (t, $J = 2.4$ Hz, 1H), 2.15-2.11 (m, 1H), 1.57-1.54 (m, 4H), 1.40 (t, $J = 5.2$ Hz, 1H), 1.29 (s, 3H). HRMS calculated for $C_{19}H_{21}ClN_6O_3$ ($M + H$)⁺: 416.1364; found 416.1374.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-(prop-2-ynylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (31)—10% Trifluoroacetic acid (3 mL) was added to a solution of compound **30** (70 mg, 0.167 mmol) in methanol (4 mL) and heated at 70 °C overnight. Solvent was evaporated under vacuum, and the residue was purified on flash silica gel column chromatography (CH₂Cl₂:MeOH = 40:1) to give compound **31** (55 mg, 87%) as a syrup. ¹H NMR (CD₃OD, 400 MHz) δ 8.06 (s, 1H), 5.09 (d, $J = 5.6$ Hz, 1H), 4.8 (s, 1H), 4.36 (br s, 2H), 4.01 (d, $J = 6.4$ Hz, 1H), 2.88 (s, 3H), 2.63 (t, $J = 2.4$ Hz, 1H), 2.08-2.04 (m, 1H), 1.82 (t, $J = 4.8$ Hz, 1H), 1.40-1.37 (m, 1H). HRMS calculated for $C_{16}H_{18}ClN_6O_3$ ($M + H$)⁺: 377.1129; found 377.1124.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-((1-(4-fluoro-3-nitrophenyl)-1H-1,2,3-triazol-4-yl)methylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (32)—4-Fluoro-3-nitro-phenylazide (9.7 mg, 0.053 mmol) and TBTA (1 mg, 0.001 mmol) were added to a solution of compound **31** (14.4 mg, 0.038 mmol) in *t*-butanol (0.8 mL)-water (0.8 mL) at room temperature. Freshly prepared 1M solution of sodium ascorbate (38.2 μ L) followed by 7.5% solution of copper sulfate (63 μ L, 0.019 mmol) was also added into the reaction mixture stirred at room temperature overnight. Solvent was evaporated, and the residue was purified on flash silica gel column chromatography (CH₂Cl₂:MeOH = 30:1) to give compound **32** (19 mg, 92%) as a syrup. ¹H NMR (CD₃OD, 400 MHz) δ 8.62-8.61 (m, 2H), 8.27-8.24 (m, 1H), 8.06 (s, 1H), 7.69-7.64 (m, 1H), 5.08 (d, $J = 6.8$ Hz, 1H), 4.94 (br s, 2H), 4.81 (s, 1H), 4.01 (d, $J = 6.0$ Hz, 1H), 2.87 (s, 3H), 2.07-2.04 (m, 1H), 1.82 (t, $J = 4.8$ Hz, 1H), 1.39-1.37 (m, 1H). HRMS calculated for $C_{22}H_{21}ClFN_{10}O_5$ ($M + H$)⁺: 559.1369; found 559.1346.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-((1-(4-fluoro-3-amino)-1H-1,2,3-triazol-4-yl)methylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (33)—Compound **33** (73%) was synthesized from compound **32** following the same procedure as for compound **16**. ¹H NMR (CD₃OD, 400 MHz) δ 8.32 (s, 1H), 8.06 (s, 1H), 7.27-7.25 (m, 1H), 7.13-7.08 (m, 1H), 6.99-6.95 (m, 1H), 5.08 (d, *J* = 7.2 Hz, 1H), 4.82 (s, 2H), 4.00 (d, *J* = 6.8 Hz, 1H), 2.87 (s, 3H), 2.07-2.04 (m, 1H), 1.82 (t, *J* = 4.8 Hz, 1H), 1.40-1.38 (m, 1H). HRMS calculated for C₂₂H₂₃ClFN₁₀O₃ (M + H)⁺: 529.1627; found 529.1616.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-((1-(4-(3-fluoropropylcarbamoyl)phenyl)-1H-1,2,3-triazol-4-yl)methylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (34)—Compound **34** (89%) was synthesized from compound **31** following the same procedure as for compound **32**. ¹H NMR (CD₃OD, 400 MHz) δ 8.56 (s, 1H), 8.06 (s, 1H), 8.02 (d, *J* = 8.8 Hz, 2H), 7.96 (d, *J* = 8.8 Hz, 2H), 5.08 (d, *J* = 6.8 Hz, 2H), 4.95 (br s, 2H), 4.82 (s, 1H), 4.61 (t, *J* = 5.6 Hz, 1H), 4.51 (t, *J* = 5.6 Hz, 1H), 4.00 (d, *J* = 6.8 Hz, 1H), 3.54 (t, *J* = 6.8 Hz, 2H), 2.87 (s, 3H), 2.08-1.96 (m, 3H), 1.82 (t, *J* = 4.8 Hz, 1H), 1.40-1.36 (m, 1H). HRMS calculated for C₂₆H₂₉ClFN₁₀O₄ (M + H)⁺: 599.2046; found 599.2030.

(1S, 2R, 3S, 4R, 5S)-4-(2-Chloro-6-((1-(adamantyl)-1H-1,2,3-triazol-4-yl)methylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (35)—Compound **35** (95%) was synthesized from compound **31** following the same procedure as for compound **32**. ¹H NMR (CD₃OD, 400 MHz) δ 8.04 (s, 1H), 8.03 (s, 1H), 5.08 (d, *J* = 6.8 Hz, 1H), 4.82 (s, 2H), 4.01 (d, *J* = 6.4 Hz, 1H), 2.87 (s, 3H), 2.25 (s, 9H), 2.07-2.04 (m, 1H), 1.84-1.81 (m, 7H), 1.40-1.36 (m, 1H). HRMS calculated for C₂₆H₃₃ClN₉O₃ (M + H)⁺: 554.2395; found 554.2383.

(1R,2R,3S,4R,5S)-4-(2-Chloro-6-(prop-2-ynylamino)-9H-purin-9-yl) 2,3-O-isopropylidene-bicyclo[3.1.0]hexane (37)—Propargylamine (47 μL, 0.7 mmol) and triethylamine (0.28 mL, 1.9 mmol) were added to a solution of compound **36** (50 mg, 0.14 mmol) in anhydrous methanol (2 mL) and stirred overnight at room temperature. Solvent was evaporated, and the residue was purified on flash silica gel column chromatography (hexane:ethyl acetate=1:1) to give compound **37** (45 mg, 85%) as a syrup. ¹H NMR (CD₃OD, 300 MHz) δ 7.80 (s, 1H), 6.25 (br s, 1H), 5.34 (t, *J* = 5.7 Hz, 1H), 4.98 (s, 1H), 4.64 (d, *J* = 7.2 Hz, 1H), 4.48 (s, 2H), 2.03-2.12 (m, 1H), 1.78 (s, 1H), 1.62-1.67 (m, 1H), 1.54 (s, 3H), 1.25 (s, 3H), 0.88-0.98 (m, 2H). HRMS calculated for C₁₇H₁₉ClN₅O₂ (M + H)⁺: 360.1227; found 360.1218.

(1R,2R,3S,4R,5S)-4-(2-Chloro-6-(prop-2-ynylamino)-9H-purin-9-yl) bicyclo[3.1.0]hexane-2,3-diol (38)—A solution of compound **37** (40 mg, 0.11 mmol) in methanol (3 mL) and 10% trifluoromethane sulfonic acid-water (3 mL) was heated at 70 °C overnight. Solvent was evaporated, and the residue was purified on flash silica gel chromatography (CH₂Cl₂:MeOH = 50:1) to give compound **38** (33 mg, 93%) as a syrup. ¹H NMR (CD₃OD, 300 MHz) δ 8.15 (s, 1H), 4.79 (s, 1H), 4.70 (t, *J* = 6.0 Hz, 1H), 4.36 (s, 2H), 3.89 (d, *J* = 6.6 Hz, 1H), 2.62 (s, 1H), 1.95-2.01 (m, 1H), 1.62-1.68 (m, 1H), 1.28-1.32 (m, 1H), 0.72-0.79 (m, 1H). HRMS calculated for C₁₄H₁₅ClN₅O₂ (M + H)⁺: 320.0914; found 320.0898.

(1R,2R,3S,4R,5S)-4-(2-Chloro-6-((1-(4-fluoro-3-nitrophenyl)-1H-1,2,3-triazol-4-yl)methylamino)-9H-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (39)—4-Fluoro-3-nitro-phenylazide (9.88 mg, 0.054 mmol) and TBTA (1 mg, 0.001 mmol) were added to a solution of compound **38** (12.4 mg, 0.038 mmol) in *t*-butanol (0.6 mL)-water (0.6 mL) at

room temperature. A freshly prepared 1M solution of sodium ascorbate (38.7 μ L) followed by 7.5% solution of copper sulfate (51.6 μ L, 0.015 mmol) were also added to the reaction mixture, which was then stirred at room temperature overnight. Solvent was evaporated, and the residue was purified on flash silica gel column chromatography (CH_2Cl_2 :MeOH = 30:1) to give compound **39** (18 mg, 96%) as a syrup. ^1H NMR (CD_3OD , 300 MHz) δ 8.60-8.64 (m, 2H), 8.22-8.27 (m, 1H), 8.15 (s, 1H), 7.62-7.68 (m, 1H), 4.79 (s, 1H), 4.70 (t, J = 5.1 Hz, 1H), 4.37 (s, 2H), 3.88 (d, J = 6.6 Hz, 1H), 2.62 (t, J = 2.4 Hz, 1H), 1.95-1.99 (m, 1H), 1.62-1.68 (m, 1H), 1.26-1.32 (m, 1H), 0.72-0.79 (m, 1H). HRMS calculated for $\text{C}_{20}\text{H}_{18}\text{ClFN}_9\text{O}_4$ ($M + \text{H}$) $^+$: 502.1154; found 502.1150.

(1*R*,2*R*,3*S*,4*R*,5*S*)-4-(2-Chloro-6-(1-adamantyl-1*H*-1,2,3-triazol-4-yl)methylamino)-9*H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (40)—Compound **40** (92%) was prepared from compound **38** following the same method for compound **37**. ^1H NMR (CD_3OD , 300 MHz) δ 8.13 (s, 1H), 8.02 (s, 1H), 4.81-4.84 (m, 2H), 4.79 (s, 1H), 4.70 (t, J = 6.0 Hz, 1H), 3.89 (d, J = 6.0 Hz, 1H), 2.24 (s, 10H), 1.95-1.97 (m, 1H), 1.82 (s, 6H), 1.63-1.66 (m, 1H), 1.28-1.32 (m, 1H), 0.73-0.77 (m, 1H). HRMS calculated for $\text{C}_{24}\text{H}_{30}\text{ClN}_8\text{O}_2$ ($M + \text{H}$) $^+$: 497.2180; found 497.2164.

Dendrimer conjugates—Synthetic procedures for each of three dendrimer conjugates are provided below. After reaction in DMSO as specified, each conjugate was separated from small molecular weight impurities by extensive dialysis for 2 d and then lyophilized. We utilized Spectra/Por dialysis tubing, MWCO 3,500, diameter 11.5 mm (Spectrum Laboratories, Rancho Dominguez, CA, USA). Typically, 10 - 15 mg of dendrimer from the reaction mixture was to a volume of 5 - 7 mL with DMSO, transferred to the tubing, and placed in stirring water in a 1.5 L beaker. The water was changed every 2 h, 5X daily, for 3 d. Then, after lyophilization each dendrimer conjugate was redissolved in DMSO (~1 mL per 2 mg) and treated with an equal volume of aqueous sodium-EDTA solution (0.5 M) to remove the residual copper by complexation. The mixture was stirred overnight at room temperature followed by extensive dialysis for 2 d as before and lyophilization to give the desired dendrimer conjugate.

For multiple steps of ultrafiltration and biological assays, dilute solutions (1 μ M) of the dendrimer conjugates were prepared in 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl_2 buffer. Wash buffer solutions whose concentrations of Tris-HCl, MgCl_2 , and DMSO match those of the solutions of dendrimer conjugates were also prepared for rinsing the centrifugal filters prior to ultrafiltration. Centrifugal filters (Milipore Ltd., Amicon Ultra-0.5, 10000 MW Cutoff) were rinsed with 400 μ L of wash buffer for 5 min at $14,000 \times g$, after which the filters were placed into new microcentrifuge tubes.

Ultrafiltration of the solutions of dendrimer conjugates was then performed by adding 400 μ L of each solution to a filter, spinning the filters for 15 min at $14,000 \times g$, transferring the filtrate to a microcentrifuge tube for analysis in a radioligand binding assay and re-suspending the material remaining in each filter in 400 μ L of buffer. This process was repeated 8 times to ensure the complete removal of contaminants. After the 8th spin, the remaining material was collected by inverting the filters, placing them into new microcentrifuge tubes, and spinning the filters for 2 min at $1000 \times g$. Once collected, the remaining material for each dendrimer sample (approx. 20 μ L) was resuspended in 400 μ L of 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl_2 buffer. Finally, each of the fractions collected during the rinse and ultrafiltration steps and the resuspended material retained by the filter were tested in a radioligand binding assay using A_3 expressing CHO cell derived membranes to determine binding activity. This confirmed that no small molecular impurities remained following multiple ultrafiltration steps.

G4 PAMAM, Conjugated with Compound 9 and PEG (43)—Freshly prepared aqueous sodium ascorbate (1 M, 22 μ L) was added to a mixture of PEG-azide dendrimer **42** (15 mg, 0.25 μ mol, prepared as described³²) and compound **9** (5.7 mg, 10.98 μ mol) in a mixture of DMSO (0.7 mL) and water (0.7 mL), followed by addition of 7.5% aqueous cupric sulfate (146 μ L, 43.93 μ mol). The reaction mixture was stirred at room temperature overnight, and the product was purified by dialysis and EDTA treatment, as described above. The mixture was lyophilized to give compound **41** (14 mg, 72%) as a colorless foamy solid. ¹H NMR (DMSO, 400 MHz) δ 8.88 (s), 8.10 (s), 7.82 (s), 7.56 (s), 7.37 (s), 7.29 (br s), 5.42 (s), 4.93 (s), 4.82 (m), 4.66 (m), 4.35 (s), 4.13 (s), 3.32 (s), 3.10 (br s), 2.87 (br s), 2.33 (s), 2.17 (br s), 1.73 (br s), 1.60 (br s), 1.29 (br s). ESI-MS: calcd. 77,501; found 77,512.

G4 PAMAM, Conjugated with Compound 23 (45)—Freshly prepared aqueous sodium ascorbate (1 M, 90 μ L) was added to a mixture of azido dendrimer **44** (5.6 mg, 0.35 μ mol, prepared as described²⁹) and compound **23** (10.7 mg, 22.4 μ mol) in a mixture of DMSO (0.6 mL) and water (0.6 mL), followed by addition of 7.5% aqueous cupric sulfate (150 μ L, 44.8 μ mol). The reaction mixture was stirred at room temperature overnight, and the product was purified by dialysis and EDTA treatment, as described above. The mixture was lyophilized to give compound **45** (12.9 mg, 79%) as a white foamy solid. ¹H NMR (DMSO, 400 MHz) δ 8.86 (s), 8.24 (s), 7.25–7.36 (m), 5.13 (d, J = 4.4 Hz), 4.63 (br s), 4.54 (d, J = 7.2 Hz), 3.79 (br s), 2.76 (s), 2.67 (s), 2.43 (t, J = 6.4 Hz), 2.33 (br s), 2.21 (br s), 1.81 (br s), 1.59 (br s), 1.51 (br s), 1.23 (s), 1.11 (d, J = 3.6 Hz), 0.62 (br s). ESI-MS: calcd. 46,343; found 46,024.

G4 PAMAM, Conjugated with Compound 31 and PEG (46)—Compound **46** (63%) was prepared from compound **31** following the same method as for compound **43**. ¹H NMR (CD₃OD, 400 MHz) δ 8.56 (br s, 1H), 8.10–7.94 (br m), 7.56 (br s), 5.42 (br s), 4.94 (s), 4.67 (br m), 4.35 (br s), 4.12 (br s), 3.50 (br s), 3.2 (s), 2.8 (br s), 2.31 (br s), 1.82 (br s), 1.59 (br s), 1.29 (br s). ESI-MS: calcd. 71,511; found 71,413.

Pharmacology

Cell culture and membrane preparation—Chinese hamster ovary (CHO) cells stably expressing either the recombinant hA₁ or hA₃AR and human embryonic kidney (HEK) 293 cells stably expressing the human (h) A_{2A}AR were cultured in DMEM and F12 (1:1), supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 2 μ mol/mL glutamine. In addition, we added 800 μ g/mL Geneticin to the hA_{2A}AR media and 500 μ g/mL Hygromycin B to the hA₁AR, hA_{2B}AR, and hA₃AR media. After harvesting the cells, we centrifuged them at 250 $\times$ g for 5 min at 4 °C. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5), containing 10 mM MgCl₂. The suspension was homogenized with an electric homogenizer for 10 s and was then recentrifuged at 20,000 $\times$ g for 30 min at 4 °C. The resultant pellet was homogenized again, resuspended in the buffer mentioned above in the presence of 3 U/mL adenosine deaminase, finally pipetted into 1 mL vials, and this crude membrane preparation was stored at –80°C until binding experiments were conducted. The concentration of protein was determined using a BCA Protein Assay Kit from Pierce Biotechnology (Rockford, IL).³³

Radioligand binding studies—Radioligand binding assays at A₁, A_{2A}, and A₃ARs were performed according to the procedures described previously.^{34–36} Each tube in the binding assay contained 100 μ L of a crude membrane preparation in suspension (20 μ g of protein), 50 μ L of a stock solution of agonist radioligand, and 50 μ L of increasing concentrations of the test ligands in Tris-HCl buffer (50 mM, pH 7.5) containing 10 mM

MgCl₂. Nonspecific binding was determined using a final concentration of 10 μ M 5'-N-ethylcarboxamido-adenosine (NECA), a non-specific agonist, diluted with the buffer.

The mixtures were incubated at 25 °C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under a reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburg, MD). Filters were washed three times with 5 mL of 50 mM ice-cold Tris-HCl buffer (pH 7.5). The radioactive agonists [³H]R-N⁶-(phenylisopropyl)adenosine (R-PIA) **47** and [³H]2-[p-(2-carboxyethyl)phenyl-ethylamino]-5'-N-ethylcarboxamido-adenosine (CGS21680) **48** were used for the A₁ and A_{2A}AR assays, respectively, while [¹²⁵I]N⁶-(4-amino-3-iodobenzyl)adenosine-5'-N-methyluronamide (I-AB-MECA) **49** was used for the A₃AR assay. Filters for A₁ and A_{2A}AR binding were placed in scintillation vials containing 5 mL of Hydrofluor scintillation buffer and counted using a PerkinElmer Tricarb 2810TR Liquid Scintillation Analyzer. Filters for A₃AR binding were counted using a PerkinElmer Cobra II γ -counter.

Cyclic AMP accumulation assay—Intracellular cyclic AMP levels were measured with a competitive protein binding method.³⁷ CHO cells that expressed the recombinant hA₃AR were harvested by trypsinization. After centrifugation and resuspending in medium, cells were planted in 24-well plates in 1.0 mL medium. After 24 h, the medium was removed and cells were washed three times with 1 mL DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with the agonist NECA (10 μ M) and/or test compound (1 or 10 μ M) in the presence of rolipram (10 μ M) and adenosine deaminase (3 units/mL). After 30 min forskolin (10 μ M) was added to the medium, and incubation was continued for an additional 15 min. The reaction was terminated by removing the supernatant, and cells were lysed upon the addition of 200 μ L of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at -20 °C. For determination of cyclic AMP production, a cyclic AMP kit (Sigma, St. Louis, MO) was used.

Data analysis—Binding and functional parameters were calculated using Prism 5.0 software (GraphPAD, San Diego, CA, USA). IC₅₀ values obtained from competition curves were converted to K_i values using the Cheng-Prusoff equation.³⁸ Data were expressed as mean $\pm$ standard error. The concentrations of the dendrimer-ligand complexes are measured by the concentration of the dendrimer, not the ligand. Therefore, binding K_i values of the dendrimer derivative are expressed as K_{i app} values.

Molecular modeling

Homology model and ligand construction: Detailed modeling procedures and a Scheme (S2) are in the Supporting Information. Briefly, the sequence of the human A₃AR was mapped onto the crystal structure of the A_{2A}AR (minus the T4-lysozyme insertion) in complex with the agonist UK-432097³⁹ (PDB ID 3QAK)⁴⁰ as a structural template for the homology modeling of the A₃AR. The UK-A_{2A}AR X-ray structure³⁹ shows four disulfide bridges present in the EL region of this receptor, but only one is relevant to the A₃AR, i.e. a bond between Cys77 (TM3) and Cys166 (EL2) that is conserved among family A GPCRs. The conformational differences in the EL region between A_{2A}AR with its four disulfides and A₃AR with a single disulfide and their effects on ligand recognition are the subject of ongoing studies. The top ranked A₃AR model built using the Homology Model tool of Molecular Operating Environment (MOE)[#] was used for the docking of nucleosides.

[#]Molecular Operating Environment (MOE), 2010.10; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2010.

The structure of conjugate **46** was imported as a mol file into MOE, where it was subjected to energy minimization using the CHARMM27 forcefield and subjected to 230 ns of Molecular Dynamics (MD) simulations in a solvated system using Desmond,[†] as implemented in the software from Schrödinger (Cambridge, MA). The final configuration of the dendrimer-PEG conjugate **46** from the simulation in water was then used for the building of the complex A₃AR-dendrimer conjugate.

Molecular Docking: A portion of **46** containing the N⁶-triazolylmethyl (N)-methanocarba nucleoside moiety and part of the dendrimer chain (Figure S1) was minimized with the Polak-Ribiere conjugate gradient (PRCG) minimization and used as starting point for the docking study. The flexible docking of the ligand in the A₃AR model was performed by means of Glide and MacroModel in the software from Schrödinger, LLC (Cambridge, MA). The molecular docking of the compound was performed in a rigid binding site of the A₃AR model with Glide using the SP (standard precision) procedure. The best-ranked complex receptor-ligand was subjected to conformational analysis by means of MCM for further structural optimization and energy minimization. During the conformational search full flexibility was granted to the ligands and the residues within a radius of 4 Å from the ligand.

Complex of A₃AR with dendrimer conjugate **46:** Randomly, one of the N⁶-triazolylmethyl nucleoside moieties of **46** was replaced with the docked conformation of the ligand in the A₃AR binding site. The dendrimer conjugate **46** with a docked nucleoside terminal inside the A₃AR binding site was then subjected to minimization in MOE with MMFF94 forcefield to a root mean square gradient of 0.001.

RESULTS

Chemical approaches to couple ring-constrained nucleoside monomers by click chemistry through their N⁶ position to PAMAM dendrimers were explored. The effects of these structural changes on receptor recognition were examined in radioligand binding assays and functional assays of cyclic AMP. Furthermore, the binding of a representative dendrimer conjugate was visualized and specific amino acid interaction predicted with molecular modeling using an A₃AR homology model based on the recently reported X-ray structures of an agonist-bound A_{2A}AR.³⁹

Chemical Synthesis

We prepared various (N)-methanocarba adenosine derivatives (Table 1) to act as selective ligands of the A₃AR. Both small molecular model compounds (**9–17**, **22–40**) and PAMAM dendrimeric precursors and nucleoside conjugates (**41–46**) were included. There were two variations of the (N)-methanocarba ring system: either bearing a 5'-N-methyluronamide or unsubstituted, i.e. "truncated", at the 4' position. Prior to coupling the nucleosides to dendrimer carriers, we explored the SAR of alkyne-functionalized chains on the N⁶ substituent and the resultant click products, i.e. 1,2,3-triazoles. The modification of the N⁶-substituent was accomplished either through the extension of a dialkynyl chain at the 3-position of a N⁶-benzyl group with subsequent Cu(I)-catalyzed click reaction of the terminal alkyne⁴¹ as shown in Schemes 1 and 2 or by formation of an aromatic ring (i.e., 1,2,3-triazole) at a N⁶-methylene carbon (Schemes 3 and 4). The triazole was formed in the latter case from a N⁶-propargyl group combined with an azido derivative using click chemistry. The rationale for these two N⁶ variations were: either incorporation of a 3-substituted N⁶-benzyl group, which is known to enhance A₃AR selectivity, or a N⁶-triazolylmethyl group, with the expectation that this newly formed 5-membered aromatic ring would serve the same

[†]Glide, version 5.7; MacroModel, version 9.9; Maestro, version 9.2; Desmond, version 3.0; Schrödinger, LLC: New York, NY, 2010.

role in A₃AR recognition as the six-membered N⁶-benzyl ring. An arylmethyl group at the N⁶ position generally favors high affinity binding to the A₃AR with selectivity that is species-independent.

The dialkynyl chains at the 3-position of N⁶-benzyl moieties in **9** – **11** and **19** – **21** were introduced by a Sonogashira reaction⁴² at the 3-iodo position of a N⁶-benzyl ring. In the case of the 5'-N-methylcarboxamides, the 2' and 3' hydroxyl groups were unprotected during this step, i.e. reaction of derivative **4** to yield intermediates **9** – **11**. However for the truncated series, Sonogashira reactions were performed on isopropylidene-protected derivative **18** to provide intermediates **19** – **21**. Compounds **19** and **21** were isolated as pure products, but **20** was subjected to the next synthetic step without isolation. The isopropylidene groups were then removed to yield dialkyne intermediates **22** – **24**.

Then, a dialkyne that was introduced at the 3-position of a N⁶-benzyl group in 5'-methyluronamide derivatives **9** – **11** or in the truncated nucleoside series, i.e. derivatives **22** – **24**, could be subjected for further reaction with azido groups, which generally occurred exclusively at the distal alkyne. Alternately, a N⁶-propargyl group in **31** and **38** could react with an azido derivative, such as the commercially available 4-fluoro-3-nitro-phenylazide. These arylfluoride derivatives provided an additional means of chain derivatization by nucleophilic substitution.

Routes to the dendrimer conjugates involved similar click chemistry. In the case of dendrimer precursor **41**, most (56 out of 64) of the peripheral amino groups of the precursor PAMAM dendrimers were first substituted with azido groups (Scheme 5A).³² Alternately, the dendrimer precursor **44** was fully substituted with azido groups (Scheme 5B), formed using a previously reported procedure.^{29,43} Then, some of the azido groups on the dendrimer **41** were reacted with alkyne-derivatized PEG chains for water solubility to form **42**. These azido-modified dendrimers **42** and **44** were then cross-linked by click chemistry²⁸ with terminal alkynyl groups at the N⁶ position of (N)-methanocarba nucleoside derivatives. Thus, a dialkyne introduced at the 3-position of a N⁶-benzyl group in 5'-methyluronamide derivative **9** (i.e. based on full A₃AR agonists) or in the truncated nucleoside series **23** (i.e. based on partial A₃AR agonists) could be subjected for further reaction with azido groups on the dendrimer. Alternately, a N⁶-propargyl group on adenosine derivative **31** could react with the azido-derivatized dendrimer **42** (Scheme 5C). The substitution of the resulting conjugates **43**, **45**, and **46** was determined by mass spectroscopy, indicating an average of 34, 64, and 30.7 nucleoside moieties, respectively, present out of the 64 possible sites on each G4 dendrimer. Approximately 3.3 azido groups per molecule of **46** remained unreacted.

Quantification of Pharmacological Activity

We initially tested the nucleoside derivatives in binding assays at three hAR subtypes; we used standard nucleoside radioligands **47** – **49** and membrane preparations from CHO cells (A₁ and A₃) or HEK293 cells (A_{2A}) stably expressing a hAR subtype (Table 1).^{34,36,44,45} We used the previously reported agonist molecular probes of the A₃AR (**4** and **8**) for comparison in the biological assays.²³ We included the parent dendrimers **41**, which contained both azido and amino groups, and the fully azido-substituted **44** as controls; they were inactive or weakly active in inhibiting AR binding.

The small molecular ligands indicated a dependence of the AR affinity on the chain length. For example, in diyne series **9** – **11**, the affinity tended to decrease upon homologation from 3 to 5 methylenes. Compound **9** displayed a K_i of 8.1 nM at the A₃AR, and its click product **12** with 4-fluoro-3-nitro-phenylazide was also the most potent in that homologous series. Reduction of the nitroaryl group to the corresponding amine as in **16** and **17** slightly reduced

A₃AR affinity. However, the 4'-truncated dialkyne **23** was weak in AR binding, and the click products **25** – **27** were only slightly more potent. In that homologous series of 1-(4-fluoro-3-nitro-phenyl)-triazole derivatives missing a 5'-uronamide, the binding affinity at the A₃AR was independent of chain length. Therefore, the 5'-methyluronamide moiety appears to stabilize a binding mode of the nucleoside such that it interacts with the extracellular regions of the ARs in a more structure-sensitive manner.

The N⁶-propargyl intermediate **31** in the 5'-methyluronamide series was potent in binding to the A₃AR (K_i 1.5 nM) and 270-fold selective in comparison to the A₁AR. The click product **32**, a 1-(4-fluoro-3-nitrophenyl)-triazole, bound to the A₃AR with a K_i of 9.1 nM and over 100-fold selectivity, and the corresponding 3-amino derivative **33** was slightly weaker. A large 1-adamantyl group on the triazole ring of **35** was also tolerated with respect to A₃AR binding. The corresponding 4'-truncated series **38** – **40** did not achieve high AR affinity; thus, the 5'-methyluronamide was an important recognition element for the small molecules.

Two of the multivalent dendrimer derivatives, **43** and **46**, obtained through click conjugation of the corresponding 5'-methyluronamide alkynyl nucleoside precursors, retained potent binding activity at the A₃AR and displayed enhanced selectivity over the monomer precursors. Water-solubilizing PEG chains, also incorporated by click linkage, did not prevent receptor binding. The binding assays were performed on conjugate preparations subjected to 8 sequential cycles of ultrafiltration to assure that no small molecular weight contaminants were present. The N⁶-benzyl derivative **43** had a K_{i app} value of 24 nM in binding to the A₃AR, which is 3-fold less potent than the alkynyl precursor **9**, and the affinity at A₁ and A_{2A}ARs was greatly decreased. The multivalent N⁶- triazolylmethyl derivative **46** was also potent in binding to the A₃AR (K_{i app} 55 nM) and selective in comparison to the A₁AR, but it was 37-fold less potent at the A₃AR than the propargyl precursor **31**. However, the multivalent 4'-truncated N⁶-benzyl dendrimer **45** was nearly inactive in binding at the A₃AR, consistent with the weak affinity of the alkyne precursor **23**.

We tested the small molecular derivatives and nucleoside conjugates in a functional assay at the A₃AR consisting of inhibition of forskolin-stimulated cyclic AMP production in A₃AR-expressing CHO cells (Table 2). These GLiDe conjugates displayed A₃AR agonist properties with varying degrees of maximal inhibition of cyclic AMP production at 1 or 10 μM. The concentration selected was generally in >100-fold excess of the K_i value for each 5'-methyluronamide nucleoside, to approximate the maximal effect. The relative efficacy was compared to that of NECA (10 μM), taken as a reference standard. The small molecular 5'-methyluronamide derivatives **9**, **13**, **15**, **17**, and **31** – **35** displayed ≥80% of the maximal effect of NECA, indicated nearly full agonism. Other 5'-uronamide derivatives reached only intermediate efficacy despite a high affinity in A₃AR binding, e.g. terminal alkyne intermediates **10** and **11** and aryl fluorides **14** and **16**. 4'-Truncated small molecules **23**, **25** – **27** and **38** – **40** displayed low efficacy (≤27% of the maximal effect) at the A₃AR, consistent with their roughly μmolar K_i values and with the tendency of truncation to reduce A₃AR efficacy. Consistent with the binding results, only dendrimer conjugates of 5'-methyluronamide derivatives, **43** and **46**, displayed considerable activity in the functional assay. Both were full A₃AR agonists at 1 μM.

Molecular Modeling

With X-ray crystallographic structures of agonist-bound forms of the hA_{2A}AR now known,^{39,46} it is possible to extend the modeling analysis of agonist binding by homology to other AR subtypes. In the X-ray crystal structure (PDB ID: 3QAK)³⁹ of the A_{2A}AR bound to agonist UK-432097 (complex designated here: UK-A_{2A}AR), large N⁶ and C2 substituents

project toward the opening of the nucleoside binding cleft, consistent with the biological activity of dendrimer-linked conjugates.

A homology model of the human A₃AR was built using UK-A_{2A}AR as template, which was chosen among the available agonist-bound A_{2A}AR crystal structures^{39,46} because it was found to be suitable for the modeling of the binding pocket embedding other N⁶-substituted agonists. In fact, in the UK-A_{2A}AR crystal structure the conformation of the third extracellular loop (EL3), connecting TM6 and TM7, is open allowing the accommodation of the bulky N⁶ substituent of the agonist UK-432097. In the adenosine-bound or NECA-bound structures,⁴⁶ instead, EL3 appears closer to EL2 with an H-bond interaction between His264(EL3) and Glu169(EL2). The agonist-bound model of A₃AR was built with the Homology Modeling module of MOE and utilized for the initial ligand docking.

We chose for receptor docking the conjugate **46**, containing a triazole ring in place of the usual phenyl ring of the typical N⁶-benzyl derivatives, rather than the more potent conjugate **43**. Because the receptor binding of N⁶-benzyl derivatives has been modeled in previous studies,^{19,21,23,30} we wanted to probe the similarity between the two aromatic rings in their docked conformations. Initially, a fragment of **46**, i.e. a chain extended version of a N⁶-triazolylmethyl derivative (related to the 5'-methyluronamide (N)-methanocarba nucleoside series **32** – **35**) was drawn (designated compound **47**, Figure S1). After this theoretical agonist-containing terminal portion of the PAMAM conjugate was docked to the A₃AR model binding site with Glide, and the best scored docking pose was further optimized by means of a Monte Carlo Multiple Minima (MCM) conformational search of the ligand and the residues in the binding site, in order to take into account the flexibility of the receptor residues.

Separately, the model of the entire PEGylated dendrimer conjugate **46** was built and optimized by means of MD. To take into account the remainder of the PAMAM moiety of the dendrimer conjugate **46** in the receptor docking model, the extended N⁶-triazolylmethyl chain of the docked nucleoside ligand **47** was fused to a portion of the pre-optimized conjugate **46**. In other words, after the MD simulation of **46**, one of the nucleoside terminals of this dendrimer conjugate was randomly chosen and replaced by the ligand previously docked in the binding site of the A₃AR model. The model of the docked dendrimer conjugate **46** in complex with A₃AR was then subjected to further minimization in order to release possible overlays and steric clashes between the dendrimer and the receptor.

The docking pose of the functionalized nucleoside in the binding site of A₃AR model was stabilized by several H-bond and van der Waals (vdW) interactions with the residues in the binding cavity, as shown in Figure 1A. In particular, the adenine ring of the molecule was anchored to Asn250(6.55) (the number in parenthesis refer to the Ballesteros-Weinstein numbering system⁴⁷) by H-bond interactions with the N⁶ amino group and the N7 of the nucleoside. Moreover, the aromatic adenine ring of the ligand was stabilized by a π - π stacking interaction with the aromatic ring of Phe168(EL2) and favorable vdW interactions with the hydrophobic residues Leu246(6.51) and Ile268(7.39). The ribose moiety of the N⁶-triazolylmethyl nucleoside derivative was found deeper in the binding pocket, locked by H-bond interactions of the 2'- and 3'-OH groups with His272(7.43) and Ser271(7.42), respectively. The carbonyl group of the 5'-N-ethylcarboxamido moiety was H-bonded to Thr93(3.36). Other favorable vdW interactions were found between the ribose moiety and hydrophobic residues, i.e. Trp243(6.48), Leu90(3.32), Leu91(3.33), Ile98(3.40), and Ile186(5.47). The long N⁶ chain of the agonist was oriented toward the extracellular solvent, exiting the binding pocket among TM5, TM6, TM7, EL3, and EL2. In particular, the triazole moiety of the N⁶ chain was located in close proximity to Gln167(EL2),

Leu264(7.35), Ile249(6.54), Met174(5.35), Ile253(6.58), and Val169(EL2), similar to the docked position of the N^6 -benzyl ring of known A₃AR agonists (not shown).

The molecular model of the A₃AR-dendrimer conjugate complex showed how the hydrophilic PEG chains surrounded the dimethylamide moiety of the hydrophobic dendrimer (Figure 1B), possibly favoring the solubility of the conjugate. Nevertheless, the ligand moieties attached to the dendrimer were still exposed to the solvent.

DISCUSSION

We previously demonstrated that coupling of strategically functionalized A₃AR agonists to PAMAM dendrimers by click chemistry through functionalized chains attached at the adenine C2 position achieved higher affinities than coupling by amide formation.²⁹ The present study focused exclusively on coupling through the N^6 position of methanocarb nucleoside ligands using several different approaches to linkage by click chemistry. The small molecules were selected for probing the effects of sterically bulky groups at the end of a functionalized chain. Within the four separate structural series examined here, a terminal adamantyl group generally served as a suitable high affinity replacement for a substituted phenyl ring in the small model compounds, i.e. in compounds **15** and **35**. However, the same substitution in truncated nucleoside **40**, containing a shorter tether from the adenine exocyclic amine, did not preserve binding affinity. The results indicated that the N^6 position of functionalized nucleosides, like the C2 position, is also viable for the click conjugation to macromolecular carriers.

The two chemical linking strategies used here were: 1) reaction of a dialkyne moiety introduced at the N^6 position (similar to the linker chemistry used at the C2 position) and 2) formation of a triazolylmethylene moiety, intended to promote A₃AR selectivity, by the click reaction of a N^6 -propargyl group. One objective of this study was to determine if a N^6 -triazolylmethyl group formed by Cu(I)-catalyzed click chemistry could substitute for a N^6 -benzyl group in the receptor binding site. In the triazolylmethyl products, high A₃AR affinity and selectivity was maintained in the 5'-methyluronamide series, but not in the 4'-truncated series. In the case of linking through a N^6 -benzyl group, only the 5'-methyluronamide derivatives (Schemes 1 and 5A) maintained high AR affinity consistently, while the 4'-truncated derivatives were only weakly active both in the form of chain-extended small molecules and as a multivalent conjugate (Scheme 5B). Thus, truncated monomer **23** was not a suitable intermediate for dendrimeric conjugation to achieve high A₃AR affinity.

The results were found to be consistent with the recently elucidated X-ray structure of the A_{2A} receptor with a N^6 substituted adenosine derivative bound. In a docking model, the nucleoside moiety of the dendrimer conjugate **46** was found to be located deep in the binding pocket anchored by strong H-bond and hydrophobic interactions with the receptor residues, while the long chain at the N^6 position of the nucleoside is oriented toward the extracellular part of the receptor exiting to the solvent among the extracellular terminals of TM5, TM6, and TM7, between EL3 and EL2. The long PEG chains are embedding the ligand-functionalized dendrimer moiety exposing the hydrophilic monomers to the solvent and coating the PAMAM dendrimer chains. Nevertheless, the nucleoside terminals of the dendrimer conjugate are still exposed to the solvent and clearly accessible for the binding to the receptor.

A₃AR homodimers have been proposed to exist at the cell surface.⁴⁸ Whether this GLiDe conjugate participates in multivalent interaction with higher order receptor aggregates is unknown.

In addition to therapeutic applications,⁴⁹ dendrimer-based drug conjugates have been applied to biological imaging modalities, such as luminescent, fluorescent, magnetic resonance, and radioactive.^{50,51} Given the high A₃AR affinity observed in the small molecular series, there is now a possibility of introducing radioisotopes to produce new radioligands for receptor characterization in vitro and in vivo. Prosthetic groups for targeting, therapeutic applications or diagnostic purposes, such as fluorescent reporter groups, might be introduced. Several fluorinated analogues and other small molecular analogues bound to the A₃AR with high affinity and raised the possibility of their use as radioligands. This suggested the application of these structures for incorporation of the positron emitter ¹⁸F for in vivo imaging of the A₃AR.^{25,52} The fluorine in aryl amino analogues **16** and **17** would be stable toward nucleophilic displacement, while the 4-fluoro group of the corresponding nitro analogues could be readily substituted. Therefore, a radioisotope (¹⁸F) could potentially be introduced by nucleophilic fluoride attack at the nitro stage, e.g. with **12** as substrate, followed by reduction to the more stable amine. The most potent of the compounds in this series was 3-amino-4-fluoro derivative **16** with a K_i value of 14.3 nM at the A₃AR, while 3-amino-4-fluoro derivative **33** was less potent in binding.

In conclusion, we have extended the SAR of small monomeric adenosine agonists derivatized at the N⁶ position to the multivalent state. We have explored a new linking strategy to achieve the high A₃AR agonist affinity and selectivity in two PEGylated GLiDe conjugates of A₃AR-binding nucleosides from chemical, pharmacological, and structural perspectives. The structural features of the pharmacophore, the chemical nature of the linking and terminal groups of the dendrimer all modulate the biological properties of a multivalent conjugate. The success of this approach was demonstrated for G4 PAMAM dendrimers as carriers, and the application of the same triazole linkage to other dendrimer carriers, with different degrees of loading or in combination with other terminal groups, remains to be investigated.

New GLiDe conjugates of 5'-methyluronamide derivatives presented here are highly potent and selective at the A₃AR and are suitable for examination in models of autoimmune inflammatory diseases, ischemia, cancer, and other diseases subject to amelioration by A₃AR agonists. Specific combinations of receptor ligands on the same dendrimer carrier might achieve synergistic effects and prove useful for disease treatment. The in vivo properties of the two active conjugates introduced in this study could now be explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1 Abbreviations

AR	adenosine receptor
DMSO	dimethylsulfoxide
EDC	<i>N</i> -ethyl- <i>N</i> '-dimethylaminopropylcarbodiimide
GPCR	G protein-coupled receptor

cyclic AMP	adenosine 3',5'-cyclic phosphate
CGS21680	2-[<i>p</i> -(2-carboxyethyl)phenyl-ethylamino]-5'- <i>N</i> -ethylcarboxamidoadenosine
CHO	Chinese hamster ovary
Cl-IB-MECA	2-chloro- <i>N</i> ⁶ -(3-iodobenzyl)-5'- <i>N</i> -methylcarboxamidoadenosine
DMEM	Dulbecco's modified Eagle's medium
GPCR	G protein-coupled receptor
HEK	human embryonic kidney
HRMS	high resolution mass spectroscopy
NECA	5'- <i>N</i> -ethylcarboxamidoadenosine
NMR	nuclear magnetic resonance
DIPEA	diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MCMM	Monte Carlo Multiple Minima
PAMAM	polyamidoamine
PEG	polyethylene glycol
PET	positron emission tomography
R-PIA	(<i>R</i>)- <i>N</i> ⁶ -(phenylisopropyl)adenosine
TBTA	tris[(1-benzyl-1 <i>H</i> -1,2,3-triazol-4-yl)methyl] amine
TEA	triethylamine
TLC	thin layer chromatography
UK-432097	6-(2,2-diphenylethylamino)-9-((2 <i>R</i> ,3 <i>R</i> ,4 <i>S</i> ,5 <i>S</i>)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)- <i>N</i> -(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9 <i>H</i> -purine-2-carboxamide

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Figure 1.

(A) Docking pose of (N)-methanocarpa adenosine derivative **47** (structure in Figure S1), which is a fragment of conjugate **46**, in the binding site of the A₃AR model based on homology to the agonist-bound A_{2A}AR crystal structure.³⁹ The nucleoside moiety of the ligand is anchored in the binding site by key H-bond interactions (highlighted with dotted lines) with the residues Thr94 (TM3), Asn250 (TM6), and Ser271 and His272 (TM7). The ligand, in ball-and-stick representation, is depicted with carbon atoms colored in orange. The receptor residues are shown in stick model with carbons in grey. (B) A molecular model of the complex A₃AR-dendrimer conjugate **46**. The receptor is represented as a cartoon colored in light blue. The dendrimer conjugate **46** is represented by a molecular surface, with the PEG chains colored in red, the ligand moieties in green, and the PAMAM moiety in grey. The likely glycosylation of the receptor in EL2 is not included in the model. Graphic images were made with PYMOL v0.99, Delano Scientific LLC, CA, USA.

Scheme 1.

Synthesis of 5'-N-methyluronamide (N)-methanocarpa adenosine derivatives linked through a N^6 -benzyl group as model compounds.

Scheme 2.

Synthesis of 4'-truncated (N)-methanocarba adenosine derivatives linked through a N^6 -benzyl group as model compounds.

Scheme 3.

Synthesis of 5'-*N*-methyluronamide (N)-methanocarpa adenosine derivatives linked through a *N*⁶-triazolylmethyl group as model compounds.

Scheme 4.

Synthesis of 4'-truncated (N)-methanocarpa adenosine derivatives linked through a N⁶-triazolymethyl group as model compounds.

Scheme 5.

Synthesis of dendrimeric conjugates of strategically functionalized nucleoside derivatives. The derivatives contain (N)-methanocarpa adenosine derivatives linked through a N^6 -benzyl (A,B) or N^6 -triazolymethyl (C) group. A 5'- N -methyluronamide (A,C) or 4'-truncated nucleoside (B) is present. Average structures are shown.

Chart 1.

Representative nucleosides that bind to the A₃AR with high affinity and selectivity (binding K_i values in nM at the human homologue given).

Table 1

Potency of a series of (N)-methanocarba adenosine derivatives at three subtypes of human ARs.

Compd	n =	R =	AR Affinity K _i , nM or (% inhibition) ^a		
			A ₁	A _{2A}	A ₃
4^{b,c}	-		136 ± 22	784 ± 97	1.5 ± 0.2
8^{b,c}	-		3040 ± 610	1080 ± 310	1.44 ± 0.60
9	3	-C≡CH	104 ± 24	4950 ± 960	8.1 ± 3.1
10	4	-C≡CH	1490 ± 230	(19.7 ± 2.4%)	113 ± 24
11	5	-C≡CH	(47.7 ± 6.1%)	(7.0 ± 4.3%)	87.2 ± 49
12	3		37.4 ± 12.0	1960 ± 50	3.2 ± 1.1
13	4		2420 ± 230	(28.6 ± 5.7%)	71.7 ± 54
14	5		1290 ± 290	(25.2 ± 12.6%)	30.1 ± 13

Compd	n =	R =	AR Affinity K _i , nM or (% inhibition) ^a		
			A ₁	A _{2A}	A ₃
15	4		1720 ± 240	(17.0 ± 8.7%)	21.8 ± 5.3
16	3		640 ± 140	2730 ± 380	14.3 ± 2.3
17	5		3780 ± 280	550 ± 90	47.6 ± 11.3
23	4	-C≡CH	(48.5 ± 5.1%)	(44.1 ± 5.4%)	2500 ± 640
25	3		(11.0 ± 9.4%)	(16.7 ± 10.7%)	897 ± 20
26	4		(24.3 ± 3.0%)	(8.2 ± 4.6%)	933 ± 720
27	5		(29.5 ± 8.4%)	(18.0 ± 9.7%)	521 ± 250
31	-	-C≡CH	400 ± 70	(23.0 ± 1.6%)	1.5 ± 0.2
32	-		(38.0 ± 14.8%)	(19.3 ± 10.1%)	9.1 ± 1.9
33	-		1670 ± 230	(17.1 ± 2.0%)	22.6 ± 7.0
34	-		1380 ± 300	1840 ± 140	36.7 ± 4.1
35	-		(38.4 ± 0.5%)	(4.9 ± 3.6%)	38.1 ± 14.4
38	-	-C≡CH	5870 ± 830	(28.3 ± 2.8%)	302 ± 26

Compd	n	R =	AR Affinity K _i , nM or (% inhibition) ^a		
			A ₁	A _{2A}	A ₃
39	-		(44.7 ± 16%)	(19.2 ± 3.9%)	390 ± 170
40	-		(38.6 ± 5.0%)	(22.5 ± 7.3%)	2190 ± 160
41 ^d	-	Dendrimer precursor	(22 ± 18%) ^f	(29.7 ± 4.0%) ^f	(70.2 ± 3.7%) ^f
43 ^e	3	N ⁶ -Benzyl dendrimer conjugate, 5'-CONHCH ₃	(11 ± 3%) ^f	(0%) ^f	24 ± 6
45 ^e	4	N ⁶ -Benzyl dendrimer conjugate, 4'-truncated	(4 ± 2%) ^f	(0%) ^f	(42%) ^f
46 ^e	-	N ⁶ -Triazolylmethyl Dendrimer conjugate, 5'- CONHCH ₃	(8 ± 8%) ^f	(0%) ^f	55 ± 8

^a All experiments were done on CHO or HEK293 (A_{2A} only) cells stably expressing one of three subtypes of hARs. The binding affinity for A₁, A_{2A} and A₃ARs was expressed as K_i values (n = 3-5) and was determined by using agonist radioligands ([³H]R-PIA, **47**; [³H]CGS21680, **48**; or [¹²⁵I]-AB-MECA, **49**; respectively), unless noted. A percent in parentheses refers to inhibition of radioligand binding at 10 μM, unless noted. K_i app values are provided for the dendrimer derivatives **43** and **46**.

^b Values from Tchilibon et al.²¹ and Tosh et al.²³

^c **4**, MRS1898; **8**, MRS5127; **16**, MRS5362; **32**, MRS5415; **33**, MRS5519; **43**, MRS5351; **46**, MRS5439.

^d Control dendrimer structures given in Scheme 5; binding data for **41** as reported.³²

^e Nucleoside dendrimer conjugate structures given in Scheme 5.

^f Percent inhibition of radioligand binding at 1 μM.

Table 2

Maximal efficacy of (N)-methanocarba adenosine derivatives in a functional assay at the hA₃AR consisting of inhibition of cyclic AMP accumulation.

Compound	% Inhibition ^{a,b}
Cl-IB-MECA ^b	97
NECA ^b	100
4	100 ^c
8	44 ± 6 ^c
9	80.0 ± 6.4
10	57.3 ± 10.6
11	57.8 ± 21.2
12	92.0 ± 7.6
13	101 ± 8
14	64.1 ± 11.5
15	79.8 ± 4.4
16	66.7 ± 5.6
17	96.6 ± 14.3
23	7.8 ± 1.4
25	6.6 ± 2.6
26	9.9 ± 2.1
27	20.1 ± 5.2
31	103 ± 8
32	107 ± 5
33	88.3 ± 4.3
34	81.5 ± 4.8
35	106 ± 5
38	14.3 ± 0.7
39	6.4 ± 2.8
40	27.1 ± 4.3
43 ^b	86.8 ± 3.2
45 ^b	4.0 ± 2.4
46 ^b	91.3 ± 3.4

^aThe efficacy at the hA₃AR was determined by inhibition of forskolin-stimulated cyclic AMP production in AR-transfected CHO cells, as described in the text. Percent inhibition is shown, in comparison to the maximal effect of a full agonist NECA at 10 μM. Data are expressed as mean ± standard error (n = 3 - 5).

^bConcentrations used were Cl-IB-MECA and NECA at 10 μM and dendrimer derivatives **43**, **45**, and **46** at 1.0 μM, and all other compounds were determined at 10 μM. ND, not determined.

^cValues from Tosh et al.²³ **4**, MRS1898; **8**, MRS5127.