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Polyamidoamine (PAMAM) Dendrimer Conjugates of "Clickable" Agonists of the A_3 Adenosine Receptor and Coactivation of the $P2Y_{14}$ Receptor by a Tethered Nucleotide

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Abstract

We previously synthesized a series of potent and selective A_3 adenosine receptor (AR) agonists (North-methanocarba nucleoside 5′-uronamides) containing dialkyne groups on extended adenine C2 substituents. We coupled the distal alkyne of a 2-octadiynyl nucleoside by Cu(I)-catalyzed "click" chemistry to azide-derivatized G4 (fourth-generation) PAMAM dendrimers to form triazoles. A_3AR activation was preserved in these multivalent conjugates, which bound with apparent K_i 0.1–0.3 nM. They were substituted with nucleoside moieties, solely or in combination with water-solubilizing carboxylic acid groups derived from hexynoic acid. A comparison with various amide-linked dendrimers showed that triazole-linked conjugates displayed selectivity and enhanced A_3AR affinity. We prepared a PAMAM dendrimer containing equiproportioned peripheral azido and amino groups for conjugation of multiple ligands. A bifunctional conjugate activated both A_3 and $P2Y_{14}$ receptors (via amide-linked uridine-5′-diphosphoglucuronic acid), with selectivity in comparison to other ARs and P2Y receptors. This is the first example of targeting two different GPCRs with the same dendrimer conjugate, which is intended for activation of heteromeric GPCR aggregates. Synergistic effects of activating multiple GPCRs with a single dendrimer conjugate might be useful in disease treatment.

Keywords

G protein; coupled receptor; purines; alkyne; azide; radioligand binding; dendrimer

INTRODUCTION

Poly(amidoamine) (PAMAM) dendrimers are treelike polymers that have wide application in drug delivery *in vivo* (1,2). We recently reported the first example of using a PAMAM

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Supporting Information Available: ^{1}H NMR and mass spectra of representative compounds **6** and **11**. This material is available free of charge via the Internet at http://pubs.acs.org/BC.

dendrimeric scaffold for the polyvalent, nanoscale presentation of nucleosides and nucleotides to selectively activate adenosine receptors (ARs) and P2Y receptors to induce intracellular signal transduction (3–5). Both of these receptor families are cell-surface G protein–coupled receptors (GPCRs). The dendrimer carriers greatly multiply the size of the monomer agonist derivatives, and these multivalent conjugates are intended to bridge multiple binding sites present in GPCR dimers and other higher-order receptor aggregates. In general, we might expect the stability of the ligand in biological systems and its pharmacokinetic and pharmacodynamic properties to be enhanced in the conjugates in comparison to the monomeric ligands.

The A_3AR is found at various levels of expression in neutrophils, eosinophils, cardiac myocytes, skeletal muscle, astrocytes, neurons, and other cell types (6) and has distinct effects on cell survival and response to stress. The A_3AR agonist N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine (IB-MECA, CF101) **1** (Chart 1) and its 2-chloro derivative deregulate the NFκB pathway and are currently in clinical trials for the treatment of autoimmune inflammatory diseases and cancer, respectively (7–11). Anti-ischemic effects of A_3AR agonists also suggest their use in protection of the heart, lung, nervous system, and skeletal muscle (6,12,13).

We previously synthesized a series of North (N)-methanocarba nucleoside 5′-uronamides as A_3AR agonists of high affinity and selectivity (14). These selective agonists, including the anti-ischemic nucleoside MRS3558 **2**, contained a rigid bicyclo[3.1.0]hexane (methanocarba) ring system of the (N) conformation in place of the freely twisting ribose moiety (9,11,12). These agonists also contained an optimized N^6-(3-chlorobenzyl) group, which enhanced A_3AR selectivity in humans, mice, rats, and other species (14). Recently, we extended this series to include dialkyne groups on an extended adenine C2 substituent, e.g., **3a** (21). We expected the proximal alkyne to promote receptor recognition and the distal alkyne to serve as a site for coupling to reporter groups while maintaining receptor recognition. Thus, we have selected a region on the nucleoside for attachment that is distal from the pharmacophore and is predicted by modeling to extend into the extracellular region of the A_3AR (15).

Here, we coupled the distal alkyne of an A_3AR agonist containing a 2-octadiyne group to azido-derivatized G4 (fourth-generation) PAMAM dendrimer carriers with Cu(I)-catalyzed alkyne-azide cycloaddition, a type of click chemistry (16–18), to form triazole derivatives, as in conjugate **3b**, and determined the receptor-binding affinity and selectivity. This variation of click chemistry was used previously for coupling biologically active molecules to polymers (19). In our previous studies of dendrimer-bound ligands for GPCRs, the small molecular ligands were amide-linked to the carrier (3–5). For this study, we compared the amide linkages to PAMAM dendrimers with the use of azido groups for conjugation of alkyne derivatives.

We also included PAMAM dendrimers with mixed peripheral azido and amino groups in this study. The incorporation of azides for click chemistry along with the conventional terminal amino/carboxylic groups of the PAMAM dendrimers for amide coupling allowed the facile conjugation of ligands for two different GPCRs (Figure 1). We present the first example of coactivation of two GPCRs by a hybrid PAMAM dendrimer conjugate. The anti-inflammatory A_3AR and the $P2Y_{14}$ receptor, which is activated by endogenous uridine-5′-diphosphoglucose (UDPG) in the neuroimmune system (20), serve to illustrate this approach.

EXPERIMENTAL PROCEDURES

Chemical Synthesis

Materials and Methods—All reactions were carried out under a nitrogen atmosphere. We purchased *N*-ethyl-*N*′-dimethylaminopropylcarbodiimide hydrochloride (EDC.HCl), UDPG

acid trisodium salt (UDPGA), UDPG, G2.5 PAMAM (10 wt% solution in methanol), G3 PAMAM (20 wt% solution in methanol), PAMAM dendrimers (G4, G5.5, and G6, 5 wt% solution in methanol) with an ethylenediamine core from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents, except those indicated, came from Sigma-Aldrich. We purchased dialysis membranes (Spectra/Pore Membrane, MWCO 3500, flat width 18 mm) from Spectrum Laboratories (Rancho Dominguez, CA). Synthesis of compound **3a** is described elsewhere (21).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-600 spectrometer with D_2O as a solvent. The chemical shifts are expressed as relative ppm from HOD (4.80). We performed electrospray ionization mass spectrometry (MS) and matrix-assisted laser desorption ionization (MALDI) time-of-flight MS experiments on a Waters LCT Premier mass spectrometer at the Mass Spectrometry Facility, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), NIH. We analyzed the azido dendrimer precursors on a Varian 500 MHz NMR and on a Bruker Vertex 70 FT-IR. Galbraith Laboratories (Knoxville, TN) performed the elemental analysis.

G4 PAMAM, Conjugated with 6-heptynoic acid (15)—We added freshly prepared aqueous sodium ascorbate (1 M, 42 μL, 42.1 μmol) to a mixture of G4 PAMAM dendrimer **13** (6.74 mg, 0.42 μmol) and 6-heptynoic acid (1.33 mg, 10.5 μmol) in a mixture of *t*-butanol (0.3 mL) and water (0.3 mL), then added 7.5% aqueous cupric sulfate (70 μL, 20.8 μmol). The reaction mixture was stirred at room temperature overnight, and the product was purified by dialysis in water. The mixture was lyophilized to yield compound **15** (5.45 mg, 68%) as a white foamy solid. MALDI-MS: calcd. 19,029; found 18,928.

G4 PAMAM, Conjugated with A_3AR Agonist 3a and 6-heptynoic Acid (7)—We added freshly prepared aqueous sodium ascorbate (1 M, 31 μL, 31 μmol) to a mixture of compound **15** (5.45 mg, 0.28 μmol) and compound **3a** (4.13 mg, 7.74 μmol) in a mixture of *t*-butanol (0.3 mL) and water (0.3 mL), then added 7.5% aqueous cupric sulfate (51 μL, 14.3 μmol). The reaction mixture was stirred at room temperature overnight, and the product was purified by dialysis in water. The mixture was lyophilized to yield compound **7** (5.8 mg, 60%) as a white foamy solid. ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.51 (s), 8.14 (s), 7.61 (s), 7.31–7.41 (m), 5.46 (s), 5.68–5.95 (m), 3.86 (s), 2.66 (s), 2.21 (s), 1.8 (s), 1.61 (bs), 1.11–1.29 (m). MALDI-MS: calcd. 33,852; found 34,088.

G4 PAMAM, Conjugated with A_3AR Agonist 3a (6)—We followed the procedure used for compound **7** to synthesize compound **6** (69%) from **3a**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.50 (bs), 8.15 (bs), 7.60 (s), 7.29–7.59 (m), 5.44 (s), 4.67–5.05 (m), 4.16 (s), 3.86 (s), 2.78 (s), 2.67 (s), 2.08–2.23 (m), 1.82 (s), 1.61 (s), 0.98–1.30 (m). MALDI-MS: calcd. 49,991; found 50,844.

G4 PAMAM, Conjugated with UDPGA (17)—We added EDC.HCl (12.5 mg, 65.2 μmol) to a solution of compound **5** (15.3 mg, 1.01 μmol) and UDPGA trisodium salt **16** (22.9 mg, 35.43 μmol) in dimethylsulfoxide (DMSO), then added 0.1 M 2-(*N*-morpholino)ethanesulfonic acid buffer (140 μL). We used 0.1 M HCl to adjust the pH of the reaction mixture to 4.5–5.0. The reaction mixture was stirred at room temperature overnight, and the product was purified by extensive dialysis followed by lyophilization to yield compound **17** (22 mg, 64%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.19 (s), 7.86–8.14 (m), 6.55 (m), 5.80 (s), 5.67 (s), 5.38–5.47 (m), 4.07 (s), 3.97 (s), 3.63 (br s), 3.25 (s), 3.12 (s), 2.91 (s), 2.69 (s), 2.24 (s). MALDI-MS: calcd. 33,060; found 33,036.

G4 PAMAM, Conjugated with UDPGA and A_3AR Agonist 3a (8)—We followed the procedure used for compound **7** to synthesize compound **8** (66%) from compound **17**. MALDI-MS: calcd. 50,092; found 50,610.

G2.5 PAMAM, Conjugated with A_3AR Agonist 19 (9)—We followed the procedure used for compound **17** to synthesize compound **9** (54%) from dendrimer **18** and compound **19**. ^{1}H NMR (D_2O, 600 MHz) δ 7.98 (br s), 6.88 (br s), 4.56 (br s), 3.85 (br s), 3.55 (br s), 3.45 (br s), 3.16–3.36 (m), 3.16 (br s), 3.08 (br s), 2.56–2.98 (m), 2.54 (s), 2.24–2.48 (m), 1.92 (br s), 1.68 (br s). MALDI-MS: calcd. 10,598; found 10,783.

G2.5 PAMAM, Conjugated with A_3AR Agonist 20 (10)—We followed the procedure used for compound **17** to synthesize compound **10** (67%) from dendrimer **18** and compound **20**. ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.11 (s), 7.85–8.01 (m), 7.58 (s), 7.36 (s), 7.18–7.32 (m), 5.06–5.21 (m), 4.88–4.93 (m), 4.65 (s), 4.59 (s), 3.86–3.91 (m), 3.07 (s), 2.65 (s), 2.39 (s), 2.21 (s), 1.85 (s), 1.74 (br s), 1.59 (br s), 1.21–1.33 (m), 1.03 (br s). MALDI-MS: calcd. 8370; found 8369.

G3 PAMAM, Conjugated with A_3AR Agonist 22 (11)—We followed the procedure used for compound **17** to synthesize compound **11** (71%) from dendrimer **21** and compound **22**. ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.88 (s), 8.11 (s), 7.73–8.05 (m), 7.58 (s), 7.32 (s), 7.13–7.28 (m), 5.43 (s), 5.03–5.21 (br s), 4.87–4.96 (s), 4.85 (br s), 4.67 (s), 4.61 (s), 3.89 (s), 3.06 (s), 2.82 (s), 2.65 (s), 2.34–2.46 (m), 2.19 (s), 1.83 (s), 1.69–1.78 (m), 1.61 (s), 1.29 (s). MALDI-MS: calcd. 13,694; found 13,304.

G4 PAMAM, Conjugated with A_3AR Agonist 24 (12)—We added HATU (4.67 mg, 12.28 μmol) to a mixture of compound **24** (6.72 mg, 12.35 μmol) and dendrimer **23** (2.73 mg, 12.28 μmol) in dry DMF (0.5 mL), then added DIPEA (2.15 μL, 12.22 μmol). The reaction mixture was stirred at room temperature overnight, extensively dialyzed with water, then lyophilized to yield compound **12** (5.5 mg, 61%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.46 (br s), 8.10 (s), 7.79–7.93 (m), 7.60 (s), 7.27–7.36 (m), 5.45 (s), 4.67–4.93 (m), 3.87 (s), 3.08 (s), 2.86 (s), 2.66 (s), 2.41 (s), 2.19 (s), 1.78 (s), 1.61 (s), 1.23 (t, J = 24 Hz), 0.80–0.89 (m). MALDI-MS: calcd. 47,495; found 47,985.

Imidazole-1-sulfonyl azide hydrochloride (26)—A modification of a published procedure was used (22). A suspension of 6.53 g NaN_3 (**25**, 100 mmol, Aldrich, >99.5%) and 100 mL acetonitrile (Aldrich, 99.8% anhydrous) in a 500-mL round-bottom flask was cooled in an ice water bath under a flow of nitrogen. We then added 8.15 mL sulfuryl chloride (100 mmol, TCI American, >98.0%) in 20 mL) anhydrous acetonitrile drop-wise to the ice-cooled suspension. The mixture was stirred overnight at room temperature, after which we cooled the white suspension to ~0°C with ice water and added 13.03 g imidazole (191 mmol, TCI America, >98.0%). We warmed the resulting white slurry to room temperature, then stirred it for 3 h. We then diluted it with ethyl acetate (200 mL) and separated and discarded the aqueous layer. We washed the organic layer twice with deionized water (2 × 100 mL), then twice with saturated aqueous $NaHCO_3$ (2 × 100 mL). We then dried it over anhydrous $MgSO_4$ overnight and filtered it. We added an ethanolic HCl solution (196 mmol) drop-wise to the filtrate while stirring at ice-water temperature. After we added ~3 mL, a large amount of white solid appeared. We kept the mixture in an ice bath for 15 min, then at room temperature for 30 min, after which we filtered it. We washed the filter cake with ethyl acetate (3 × 100 mL) to give **26** as a colorless solid (8.65 g, 49.9%), m.p. 99–101°C. IR (KBr) 2173, 1383 and 1160 cm^{-1}. ^{1}H NMR (500 MHz, D_2O) d 7.61 (dd), 8.02 (dd), 9.39 (dd); ^{13}C NMR (125. MHz, D_2O) δ = 120.0, 123.5, 137.6. Element Analysis: Calculated C, 17.19; H, 1.92; N, 33.41. Found: C, 17.38; H, 1.98; N, 32.97.

Synthesis of Fully Azido-Derivatized PAMAM (G4) (13)—We added anhydrous methanol (10 mL, Aldrich, >99.8%) and 18.15 g G4 PAMAM dendrimer (**23**, Dendritech, Midland, MI, 14.93 w/w% in methanol, [NH_2]=12.2 mmol) to a 250-mL round-bottom flask. We then added 3.12 g K_2CO_3 (Aldrich, >99.0%, 22.6 mmol) and 31.4 mg $CuSO_4.5H_2O$ (Aldrich, 98.0%, 0.126 mmol), causing the solution to take on a pale bluish color. We next added 2.73 g imidazole-1-sulfonyl azide hydrochloride (**26**, 13.1 mmol) to the reaction mixture, and it was stirred at room temperature overnight. The mixture was then dialyzed against deionized water for 7 d, during which we changed the water every 2 h for the first 2 d and thereafter every 4 h. After lyophilization, we collected 2.51 g azide-derivatized G4 dendrimer. The proton NMR is shown in Figure 2. Element analysis: PAMAM-G4: calculated: C, 52.81; H, 8.48; N, 24.70, O, 14.01; fully azide-derivatized G4 PAMAM calculated: C, 47.03; H, 7.15; N, 33.31, O, 12.49; Found: C: 49.91; H: 7.55; N: 28.07, O: 13.12. The element analyses indicated that 98.0%of primary amines were replaced with azides.

Synthesis of Partially Azido-Derivatized PAMAM (G4) (5)—We added anhydrous methanol (5 mL, Aldrich, >99.8%) and 5.13 g G4 PAMAM dendrimer (**23**, Dendritech, 14.93 w/w% in methanol, [NH_2]=3.45 mmol) to a 100-mL round-bottom flask. We then added 0.803 g K_2CO_3 (Aldrich, >99.0%, 5.81 mmol) and 17.2 mg $CuSO_4.5H_2O$ (Aldrich, 98.0%, 0.069 mmol); the solution took on a pale-bluish color. Next we added 0.43 g imidazole-1-sulfonyl azide hydrochloride (**26**, 2.05 mmol) to the reaction mixture, which was stirred at room temperature overnight. We then dialyzed the mixture against deionized water for 7 d, changing the water every 2 h during the first 2 d and every 4 h thereafter. After lyophilization, we collected 0.45 g partially azide-derivatized G4 dendrimer. Elemental analysis of the partially azide-derivatized G4 PAMAM found C: 49.91 H: 7.55; N: 28.07; O: 13.12. We compared results of the element analysis with the composition of the G4 PAMAM dendrimer and found that 47.2% of the primary amines were replaced with azides, corresponding to ~31 amines per dendrimer in 64 terminal positions.

Biological Methods: $P2Y_{14}$ Receptor

Materials—We purchased 3-isobutyl-1-methyl-xanthine from Sigma-Aldrich and [^{3}H] adenine from American Radiolabeled Chemicals (St. Louis, MO). We obtained all cell culture medium and serum from Gibco(Invitrogen, Carlsbad, CA).

Cell culture—We generated C6 glioma cells stably expressing the human $P2Y_{14}$-R ($P2Y_{14}$-C6 cells) as previously described by Fricks et al. (23). We cultured $P2Y_{14}$-C6 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 1% Geneticin (Gibco), and 1% antibiotic-antimocotic (Gibco) at 37°C in a 5% CO_2 environment.

Accumulation of adenosine 3′, 5′-cyclic phosphate (cAMP)—We grew $P2Y_{14}$-C6 cells in 24-well plates and incubated them with 1 μCi [^{3}H]adenine/well in serum-free DMEM for at least 2 h prior to assay. We initiated the assays by adding HEPES-buffered, serum-free DMEM containing 200 μM 3-isobutyl-1-methyl-xanthine and 30 μM forskolin, with or without drugs, and incubation continued for 15 min at 37°C. We terminated incubations by aspirating the medium and adding 450 μL ice-cold 5% trichloroacetic acid. We isolated [^{3}H]cAMP by sequential Dowex and alumina chromatography and quantified it by liquid scintillation counting as previously described by Harden et al. (24).

Data analysis—We measured the concentrations of the dendrimer-ligand complexes by the concentration of the dendrimer, not the attached ligand. We determined EC_{50} and apparent K_i values with Prism software (GraphPad, San Diego, CA); they are presented as mean ± SE. The number of nucleoside moieties on a given dendrimer that are accessible to receptors is

uncertain; the data are therefore shown as apparent K_i values. We repeated all experiments at least three times.

Biological Methods: ARs

Receptor-binding and functional assays—We obtained [^{3}H]adenosine-5′-*N*-methyluronamide ([^{3}H]NECA, 42.6 Ci/mmol) from PerkinElmer Life and Analytical Science (Boston, MA). We purchased [^{3}H](2-[p-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamido-adenosine) ([^{3}H]CGS21680, 40.5 Ci/mmol) and [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]I-AB-MECA, 2200 Ci/mmol) from PerkinElmer. We prepared test compounds as 5-mM stock solutions in DMSO and stored them frozen at −20°C.

Cell culture and membrane preparation—We cultured Chinese hamster ovary (CHO) cells stably expressing the recombinant hA_1 and hA_3 and human embryonic kidney (HEK) 293 cells stably expressing the hA_{2A}AR in DMEM and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. In addition, we added 800 μg/mL Geneticin to the A_{2A} media and 500 μg/mL hygromycin to the A_1 and A_3 media. After harvesting the cells, we homogenized them with an electric homogenizer for 10 sec, pipetted them into 1-mL vials, and then stored them at −80°C until we conducted the binding experiments. We measured the protein concentration with a BCA Protein Assay Kit from Pierce Biotechnology (Rockford, IL) (25).

Binding assays—Each tube in the binding assay contained 50 μL of increasing concentrations of the test ligand in Tris-HCl buffer (50 mM, pH 7.5) containing 10 mM $MgCl_2$, 50 μL of the appropriate agonist radioligand, and 100 μL membrane suspension, added sequentially. For the A_1AR (20 μg protein/tube), we used the radioligand [^{3}H]NECA (~3.5 nM, precise final concentration is calculated for each experiment). For the A_{2A}AR (20 μg/tube), we used the radioligand[^{3}H]CGS21680 (~10 nM). For the A_3AR (21 μg/tube), we used the radioligand [^{125}I]I-AB-MECA (~0.3 nM). We determined nonspecific binding with a final concentration of 10 μM unlabeled NECAdiluted with the buffer. We incubated the mixtures at 25°C for 60 min in a shaking water bath. We terminated binding reactions by filtration through Brandel GF/B filters under a reduced pressure with a M-24 cell harvester (Brandel, Gaithersburg, MD). We washed filters three times with 3 mL 50-mM ice-cold Tris-HCl buffer (pH 7.5). We then placed filters for A_1 and A_{2A}AR binding in scintillation vials containing 5 mL Hydrofluor scintillation buffer and counted with a PerkinElmer Liquid Scintillation Analyzer (Tri-Carb 2810TR). We counted filters for A_3AR binding with a Packard Cobra II γ-counter (PerkinElmer). We determined the K_i values with GraphPad Prism for all assays.

Accumulation of cAMP—To determinecAMP production (26), we incubated protein kinase A with [^{3}H]cAMP (2 nM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150 mM; EDTA, 10 mM), 20 μL cell lysate, and 30 μL 0.1 M HCl or 50 μL cAMP solution (0–16 pmol/200 μL for standard curve). We separated bound radioactivity by rapid filtration through Whatman GF/C filters and washed the filters once with cold buffer. We measured bound radioactivity with liquid scintillation spectrometry.

RESULTS

Chemical Synthesis

We prepared various PAMAM dendrimer derivatives of nucleosides and nucleotides (Table 1, 6–**12**) to act as multivalent ligands of GPCRs. The peripheral groups of the precursor dendrimers contained either all azido groups (Scheme 1), mixtures of azido and amino groups (Scheme 2), or the conventional all amino/carboxylate groups (Scheme 3). The synthetic

methods used to partially or fully substitute terminal amino groups of a G4 PAMAM dendrimer with azides are shown in Scheme 4 (22).

We recently introduced terminal alkynyl groups at the C2 position of (N)-methanocarba nucleoside derivatives to serve as sites on the A_3AR agonists for cross-linking by click chemistry (21). Thus, a dialkyne was introduced at the C2 position in **2**, and further reaction with azides generally occurred exclusively at the distal alkyne. Prior to coupling the nucleosides to dendrimer carriers, we explored in detail the structure-activity relationship at the distal region of an alkyne-functionalized chain at the 2 position and the resultant click products (21).

The synthetic route to the multivalent dendrimer conjugates of nucleosides for activation of the A_3AR that contain 5′-*N*-methyluronamide (N)-methanocarba 2-alkynyl triazoles **6** and **7** is shown in Scheme 1. Click chemistry efficiently coupled the A_3AR agonist **3a** to a PAMAM dendrimer fully substituted with peripheral azido groups. We used two versions of this azido-functionalized dendrimeric scaffold: **15**, containing extra appended carboxylic acid groups for increasing water solubility, and **13**, lacking these groups. We added the carboxylic acid moieties to the azido dendrimer **13** by partial reaction of the peripheral azido groups with hexynoic acid **14**. Thus, we prepared two nucleoside-containing dendrimer derivatives **6** and **7**. Derivative **6** was fully substituted with the adenosine analogue, with 64 nucleoside moieties per dendrimer molecule. Derivative **7** was partially (28 out of 64) substituted with the adenosine analogue and contained 24 carboxylic acid moieties. 12 azido groups per molecule of **7** remained unreacted.

In the next phase of our study, we combined different GPCR ligands, i.e., the A_3AR and another receptor, on the same dendrimer carrier. We selected the $P2Y_{14}$ receptor, which is activated by naturally occuring uridine-5′-diphosphoglucose (UDPG), for this purpose because, like the A_3AR, it occurs on immune cells and because we recently introduced high-potency dendrimer conjugates for this subtype (5,27). Each ligand would provide selectivity for a particular GPCR subtype, and the overall selectivity of the conjugate would be a function of the union of the sets of selectivity of the attached nucleosides. We sequentially coupled alkyne and carboxylic acid derivatized ligands for the two receptors to a novel multifunctional PAMAM dendrimer. Thus, alkynes reacted exclusively with azido terminal groups and carboxylic acids with terminal amino groups on the same dendrimer to randomly (but in a defined ratio) produce a multivalent, binary conjugate.

We proceeded to synthesize a multivalent, binary conjugate of a mixed azido/amino dendrimer **5**, as shown in Scheme 2. In the dendrimer precursor **5**, we determined, through NMR (28) (Figure 2) and elemental analysis, that the ratio of amines to azides was ~31:33. Therefore, of the 64 terminal groups on each G4 dendrimer, ~33 would be available for cycloaddition, and the remaining terminal groups would be available for amide formation. We first coupled the amino groups of **5** to the functionalized, $P2Y_{14}$-selective nucleotide UDPGA **16**, which we previously showed to be a suitable functionalized congener for chain attachment without loss of receptor activation (5). We then coupled the azido groups of **17** to the functionalized nucleoside **3a**, which is a suitable functionalized congener for chain attachment without loss of binding affinity at the A_3AR (21). The product of this second coupling reaction was the mixed dendrimer **8**, in which the average ratio of content of UDGPA and nucleoside moieties was ~31:33 per G4 dendrimer. Thus, conjugate **8** would be expected to potently activate both A_3 and $P2Y_{14}$ receptors and perhaps maintain selectivity over other ARs and P2Y receptors.

To compare alkyne-azide click chemistry with the more standard amide formation as a means of linking A_3AR-selective agonists to PAMAM dendrimers, we prepared various dendrimer derivatives starting with our previously reported amino and carboxylic-functionalized

congeners **19**, **20**, **22**, and **24** (14,15). The reaction sequences are shown in Scheme 3. We included a variety of dendrimer sizes and sites of conjugation on the nucleoside.

The synthesis of the PAMAM dendrimer intermediate **5** containing equiproportioned peripheral azido and amino groups for conjugation of multiple ligands is shown in Scheme 4. We used the reagent imidazole-1-sulfonyl azide hydrochloride **26** for the conversion of peripheral amino groups to azido groups (22). We prepared a fully azido-derivatized G4 dendrimer **13** similarly and used it to synthesize conjugates **6** and **7**.

Quantification of Pharmacological Activity

We tested the dendrimer nucleoside conjugates similarly in binding assays at three hAR subtypes; we used standard radioligands (29–31) and membrane preparations from CHO cells (A_1 and A_3) or HEK293 cells (A_{2A}) stably expressing a hAR subtype (Table 1) (32,33). We used the previously reported agonist molecular probes of the A_3AR (1,2, **3a**, and 4) for comparison in the biological assays (15,21).

We included the parent dendrimer **5**, which contained both amino and azido groups, as a control; it was inactive in AR binding at 1 μM. We considered compound **4a** to be a model compound for the attachment of the 2-octadiyne derivative **3a** to azido-bearing PAMAM dendrimers by click chemistry. As reported previously, the triazole-containing monomer **4a** maintained both high potency and selectivity (at least 100-fold) for the A_3AR (21).

The multivalent dendrimer derivatives **6** and **7** obtained from **3a** through click conjugation were considerably more potent than **4a** in binding assays at the A_1, A_{2A}, and A_3 ARs. The affinity of **6** and **7** in binding to the A_3AR was particularly high, with K_i values of 0.1–0.3 nM (Figure 3). The fully substituted dendrimer **6** was only 2.3-fold more potent at the A_3AR than the less heavily loaded **7**, which was substituted with nucleosides at 44% of the theoretically available terminal positions. The selectivity of these dendrimer conjugates for the A_3AR in comparison to the A_1AR was still present (161- and 110-fold for **6** and **7**, respectively) but diminished in comparison to the monomer **4a** (>500-fold). However, the A_3AR selectivity of **6** (230-fold) and **7** (180-fold) in comparison to the A_{2A}AR was similar to that of **4a**. Thus, the presence of the carboxylic acid chain in **7** had only minor effects on the observed AR affinity and selectivity, but it increased the observed aqueous solubility. Both **6** and **7** were readily soluble in DMSO at a concentration of 1 mM, but when we diluted the DMSO stock solutions with phosphate-buffered saline for a final calculated concentration of 30 μM, we observed some cloudiness in **6** but not in **7**.

Dendrimer derivative **8** contained an additional covalently bound GPCR ligand, a nucleotide designed to activate the $P2Y_{14}$ receptor. We found that combining strategically functionalized nucleosides and nucleotideson the same dendrimer carrier —for example, to activate both adenosine and P2Y receptors—provided high-affinity recognition at both GPCRs. However, the K_i value of **8** in binding to the A_3AR (39.5 nM) indicated that it was considerably less potent than the conjugates **6** and **7**, which contained only the A_3AR agonist ligand. The A_3AR selectivity of **8** was only 2-fold in comparison to the A_1AR and 15-fold in comparison to the A_{2A}AR. Thus it was much less A_3AR selective than were **6** and **7**.

A functional assay of adenylate cyclase inhibition carried out in C6 cells expressing the human $P2Y_{14}$ receptor demonstrated that potency at this receptor was enhanced in the dendrimer conjugate **8** over that of the monomer, with an EC_{50} value of 2.24 ± 0.80 nM (Figure 4). Both UDPG and UDPGA displayed potencies in the same assay of 200–300 nM (5). This degree of potency enhancement at the $P2Y_{14}$ receptor was consistent with previous results of conjugation of UDPGA derivatives to PAMAM carriers (5). Dendrimer **17**, which contained only the ligand of the $P2Y_{14}$ receptor (i.e., 31 nucleotides bound per G4 dendrimer) and not the A_3AR-

activating moiety, displayed an EC_{50} value at the $P2Y_{14}$ receptor of 2.41 ± 0.77 nM. Curiously, nucleotide-containing dendrimers **8** and **17** also interacted with at least one other subtype of P2Y receptor, but nevertheless with selectivity for the $P2Y_{14}$ receptor. Dendrimers **8** and **17** displayed EC_{50} values in activation of phospholipase C (PLC) by the human $P2Y_2$ receptor of 1.39 ± 0.54 μM and 733 ± 223 nM (n = 3), respectively, assayed as described in stably transfected 1321N1 astrocytoma cells (34). The native agonist of the $P2Y_{14}$ receptor, UDPG, also activated the human $P2Y_2$ receptor, with an EC_{50} value of 10 μM (34).

PAMAM dendrimer conjugates (9–12) of carboxylic- and amino-functionalized congeners of A_3AR-selective agonists consistently displayed lower affinity in A_3AR binding than did the click-linked dendrimers **6** and **7**. The amide-linked conjugates included derivatives of both terminal carboxylic acid–functionalized dendrimers **9** and **10** and terminal amine-functionalized dendrimers **11** and **12**, ranging from generation 2.5 to 4. The affinities of these dendrimers at the hA_3AR were in the range of 12–42 nM, and the nature of the residual terminal groups of the dendrimer carrier had little effect. We did not observe the subnanomolar affinity seen in click-linked nucleoside-dendrimer conjugates. Thus, the high affinity is partially the result of the linkers and the coupling chemistry used, which in **6** and **7** produce a triazole rather than an amide linkage.

The selectivity of the amide-linked dendrimers in comparison to the A_{2A}AR was high (×-fold, for compound): >300, **9**; ≫24, **10**; 327, **11**; 62, **12**. However, there was a substantial gain in affinity at the A_1AR over the corresponding monomeric nucleosides. Thus, **10** and **11** lost selectivity for the A_3AR in comparison to the A_1AR. Compound **12** was reversed in its selectivity (6-fold more potent at the A_1AR than at the A_3AR). Conversely, we considered compound **4b** to be a model nucleoside derivative for the amide-linked dendrimer derivatives **9** and **12,** and it was clearly A_3AR selective. Compound **4c** (63-fold selective for the A_3AR versus the A_1AR) was a model for N^6-functionalized dendrimer conjugates **10** and **11**, although it was a higher homologue with 4 methylenes. Thus, both model compounds **4b** and **4c** were more potent and selective in binding to the A_3AR than were the corresponding amide-linked dendrimers.

We determined binding at the rat A_3AR expressed in CHO cells for selected compounds. The K_i values of the N^6-chain derivatized, amide-linked dendrimers **10** and **11** were 62.0 ± 36.5 and 4.94 ± 1.96 nM (n = 3), respectively. The triazole-linked adenosine conjugate **7** with water-solubilizing groups displayed a K_i value of 0.08 ± 0.02 nM in binding to the rat A_3AR. Thus, there was a relatively low degree of species-dependent variation in the observed A_3AR affinity (4-fold greater in rat than human).

We tested the nucleoside conjugates in a functional assay at the A_3AR (inhibition of forskolin-stimulated cAMP production in A_3AR-expressing CHO cells) as shown in Table 2. These multivalent derivatives displayed A_3AR agonist properties with varying degrees of maximal inhibition of forskolin-stimulated cAMP production at 0.1–10 μM (concentration selected to be in >100-fold excess of the K_i value for each). In general, the relative efficacy was similar to that of NECA (1 μM), taken as a reference standard. The dendrimer control was inactive. The EC_{50} value of the amide-linked adenosine conjugate **11** was 2.93 ± 0.37 nM, determined with a full curve in CHO cells stably expressing the human A_3AR (Figure 5). Conjugate **11** was a full agonist in the A_3AR-mediated inhibition of cAMP production. We also determined EC_{50} values for cAMP inhibition with full curves for the triazole-linked adenosine conjugate with water-solubilizing groups **7** (0.12 nM) and **8** (1.36 nM).

DISCUSSION

We used the Cu(I)-catalyzed alkyne-azide click reaction for linking functionalized congeners of AR ligands (21,35) to other moieties, specifically PAMAM dendrimer derivatives. In this study, we compared different chemical strategies for tethering GPCR ligands to macromolecular carriers. Dendrimer derivatives prepared by click chemistry were particularly potent and selective in binding to the A_3AR. Multivalent nucleoside-dendrimer conjugates **6** and **7** showed much greater A_3AR affinity than the corresponding monomer, the model nucleoside **4a**. Furthermore, amide-linked nucleoside conjugates were considerably weaker than click-linked conjugates in binding to the A_3AR. This illustrates the dramatic effect that both the multivalency and the intermediate structures contained within the functionalized chain can have on interaction with the target receptor (35). A comparison with various amide-linked dendrimers showed that the triazole linkage had a general A_3AR affinity–enhancing effect. Click chemistry was previously used to link small molecules to dendrimeric structures, but ours was the first application for studying PAMAM dendrimer derivatives of small-molecular GPCR ligands. Recently, peptide ligands were conjugated to dendrimers by click chemistry for activation of a GPCR (18).

Other studies have focused on PAMAM dendrimers as reversible drug carriers, for example, to deliver anticancer drugs that dissociated at the site of action (36). Our approach derivatizes a small molecule but allows it to remain active while covalently attached to the carrier (35). The binding sites of the receptors are accessible from the extracellular medium. Thus, activation of the desired receptor would not benefit from either drug release or cellular internalization of the dendrimer. The flexible dendrimer conjugate is available to spread over the cell surface and to adopt a conformation that can bridge multiple binding sites (37).

The efficiency of the click reaction readily allowed complete substitution in the case of one of the dendrimer derivatives, i.e., **6**. In previous experiences with amide coupling of functionalized nucleosides, we encountered difficulty in the complete substitution of amine-terminated dendrimers (3,4). Click chemistry proved to be a more efficient coupling technique and allowed orthogonal reaction control over the coupling chemistry when two distinct receptor ligands were bound to the same dendrimer carrier (as in **8**).

This study presents the first example of targeting two different GPCRs with the same dendrimer conjugate, intended for activation of heteromeric aggregates of GPCRs. Each ligand provided selectivity for a particular GPCR subtype, and the overall selectivity of the conjugate was a function of the union of the sets of selectivity of the attached nucleosides. Specifically, the dendrimer conjugate **8** contained ligands for both the A_3AR and the $P2Y_{14}$ receptor. The principle of attachment of UDPG derivatives to PAMAM dendrimers to enhance potency was introduced previously (5). This bifunctional dendrimer potently activated both A_3 and $P2Y_{14}$ receptors, although not to the exclusion of other ARs.

The bifunctional dendrimer **8** and its nucleotide-dendrimer precursor **17** both activated one other P2Y receptor examined, the $hP2Y_2$ receptor, with intermediate potency. UDPG itself activates the $hP2Y_2$ receptor with an EC_{50} of 10 μM for stimulation of PLC in stably transfected 1321N1 cells (34). Therefore, the potency at both subtypes of P2Y receptors was much greater in the dendrimer conjugates than in the monomer, but the desired selectivity for the $P2Y_{14}$ receptor was maintained. Thus, both covalently tethered GPCR ligands, which were previously shown to be suitable functionalized congeners for coupling to carriers, retained their activity as the mixed dendrimer conjugate **8**. This conjugate had reduced A_3AR affinity in the presence of tethered nucleotide moieties, but not reduced $P2Y_{14}$ potency due to the tethered nucleoside moieties. This is the first example of targeting two different GPCRs with the same dendrimer conjugate, which is intended for activation of heteromeric aggregates of GPCRs.

Many examples exist of two small molecules linked with a spacer group to make binary conjugates for activation of two different GPCRs (35,38,39). Some researchers have systematically varied the length of the spacer to achieve the correct fit to the two adjacent binding sites. This multivalent approach was taken in part because of uncertainty about the geometric orientation of protomer binding sites in a GPCR dimer. Flexible dendritic carriers facilitate empirical testing of a variety of spatial orientations of the same conjugate. This obviates the need to probe the optimal geometry *a priori* of a single spacer chain to achieve simultaneous binding to two sites.

Both A_3 and $P2Y_{14}$ receptors are distributed in the immune system and appear to occur on the same cell, as was shown for RBL-2H3 mast cells (27). Further experiments will be needed to determine whether heteromers composed of A_3 and $P2Y_{14}$ receptors exist and whether this conjugate is capable of bridging the binding sites on both protomers. This approach of mixing receptor ligands on the same dendrimer carrier can be extended to many other combinations of GPCRs.

We know that GPCRs tend not to be evenly or randomly distributed over the surface of a given cell; we demonstrated this for the A_3AR expressed heterologously in CHO cells (4). In general, GPCRs may exist as complexes with other receptors and associated proteins. The receptors to which a given GPCR pairs or forms a higher-order aggregate can dramatically affect its pharmacology, and the binding of agonist to both protomers does not have equivalent pharmacological effects (40). We lack effective ligand tools for separating the effects of one receptor dimeric combination from another. This approach of mixing ligands for two different receptors (35,38,39,41) promises to be a means of achieving selectivity for receptors in a given tissue, based on association or aggregation of GPCRs, in comparison to another tissue in which the same receptor may be alternatively paired. At this early stage, we do not know the topological requirements for selectively targeting these combinations of receptors, because we are just beginning to detect their existence. The multivalent binding to other types of cell surface receptors has been explored through structural modification of the carrier polymer (42).

Multivalent conjugates of GPCR ligands provide quantitatively and qualitatively different pharmacological properties than do monomers. Prosthetic groups for targeting or therapeutic purposes or reporter groups for diagnostic purposes might be introduced. Specific combinations of receptor ligands on the same dendrimer carrier might achieve synergistic effects and prove useful for disease treatment, such as inflammatory conditions, to which the current example of adenosine and P2Y receptor ligands apply. Various combinations may be proposed for targeted therapeutic applications, but the linking strategy to achieve the desired potency and selectivity still requires development. We are working on prototypical examples that could be applied to other combinations of dendrimer-bound GPCR ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AR	adenosine receptor
DMSO	dimethylsulfoxide
EDC	*N*-ethyl-*N*′-dimethylaminopropylcarbodiimide
GPCR	G protein–coupled receptor
IBMX	3-isobutyl-1-methylxanthine
AR	adenosine receptor
cAMP	adenosine 3′,5′-cyclic phosphate
CHO	Chinese hamster ovary
IB-MECA	N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
GPCR	G protein–coupled receptor
HATU	2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium
HEK	human embryonic kidney
I-AB-MECA	2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine
MS	mass spectrometry
NECA	5′-*N*-ethylcarboxamidoadenosine
NMR	nuclear magnetic resonance
DIPEA	diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PAMAM	polyamidoamine
PLC	phospholipase C
UDPG	uridine-5′-diphosphoglucose
UDPGA	uridine-5′-diphosphoglucuronic acid.

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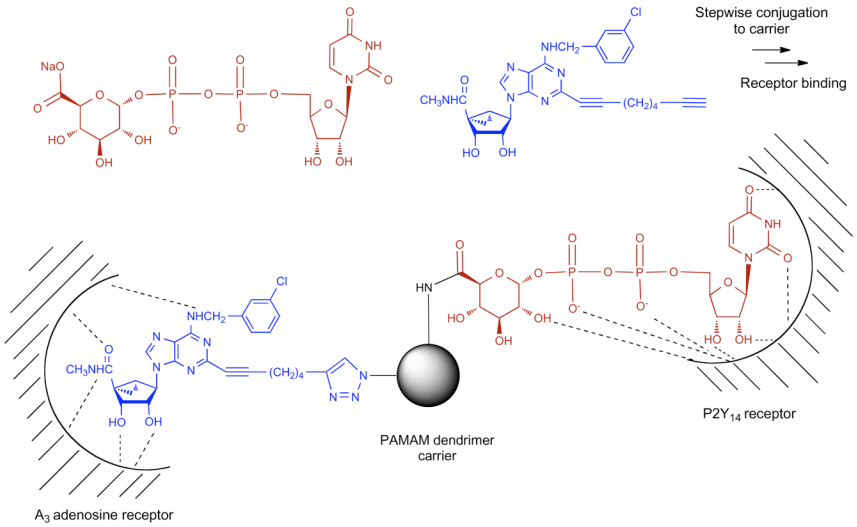


Figure 1.

Design of a bifunctional PAMAM dendrimer conjugate for the coactivation of two different GPCRs; shown here are agonists of the A_3AR and the $P2Y_{14}$ receptor. Both receptors are distributed in the immune system and may occur on the same cell (27). The stoichiometry of substitution of the dendrimer is not indicated.

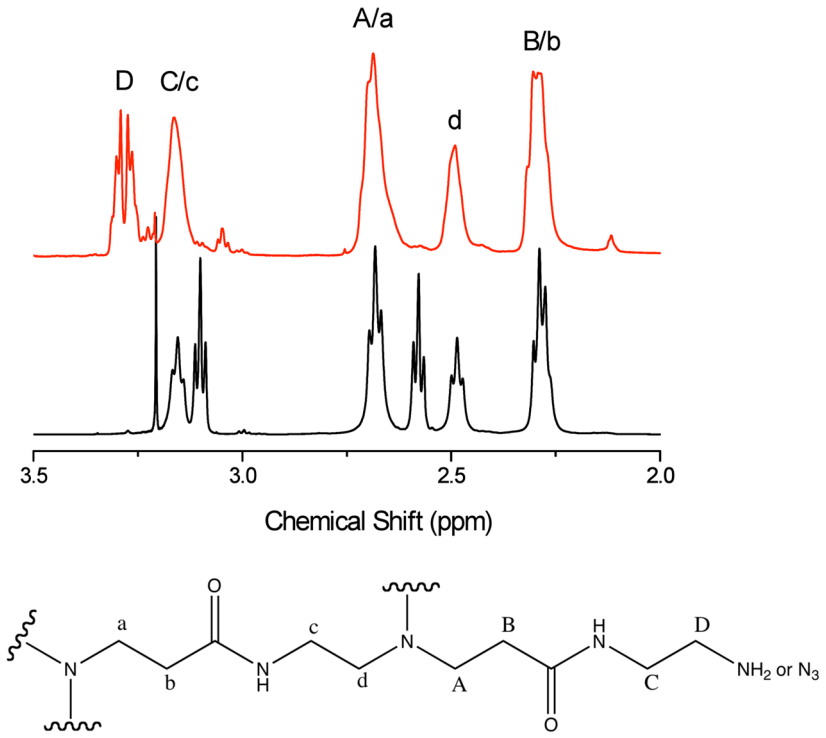
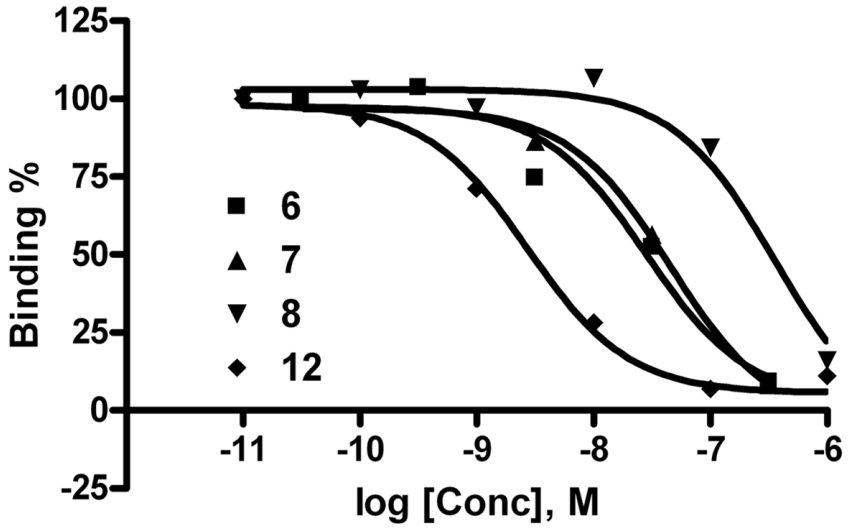


Figure 2.
^{1}H NMR spectra of G4-N_3 (**5**, top), G4-NH_2 from D_2O (**23**, middle). Schematic indication of assignments of the methylene groups (bottom).

A



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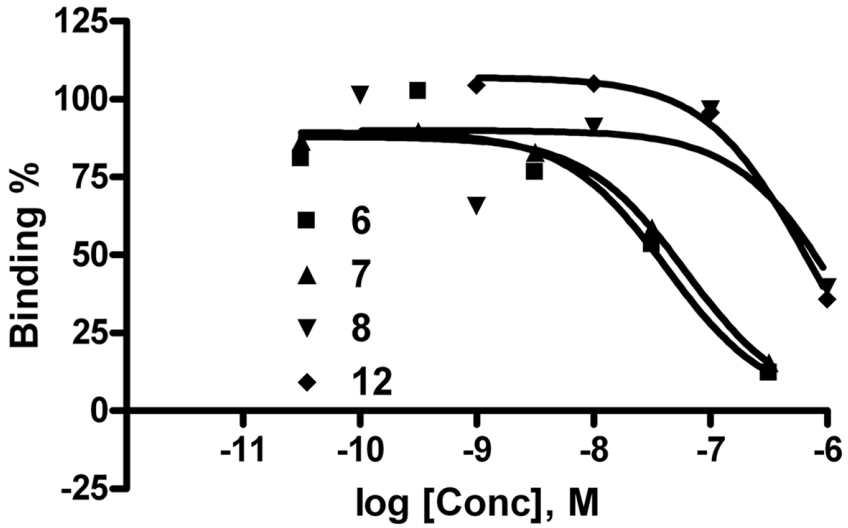

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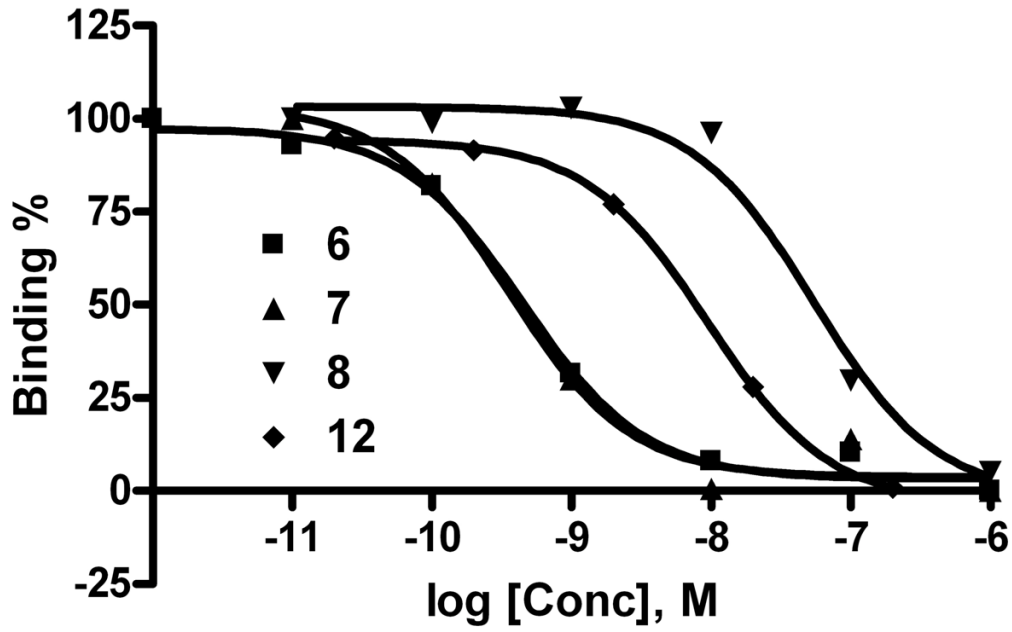


Figure 3.

Inhibition of radioligand binding by dendrimer nucleoside conjugates in membranes of CHO cells expressing the human A_1 (A) and A_3 (C) ARs and HEK-293 cells expressing the human A_{2A}AR (B). Inhibition curves at three ARs are shown for the dendrimer-nucleoside conjugates **6** and **7**, in which the linkage to an azide-derivatized dendrimer was formed by click cycloaddition, for the mixed nucleoside/nucleotide conjugate **8** and for the amide-linked nucleoside conjugate **12**. Both **6** and **7** were highly selective for the hA_3AR in comparison to the hA_1 and hA_{2A}ARs.

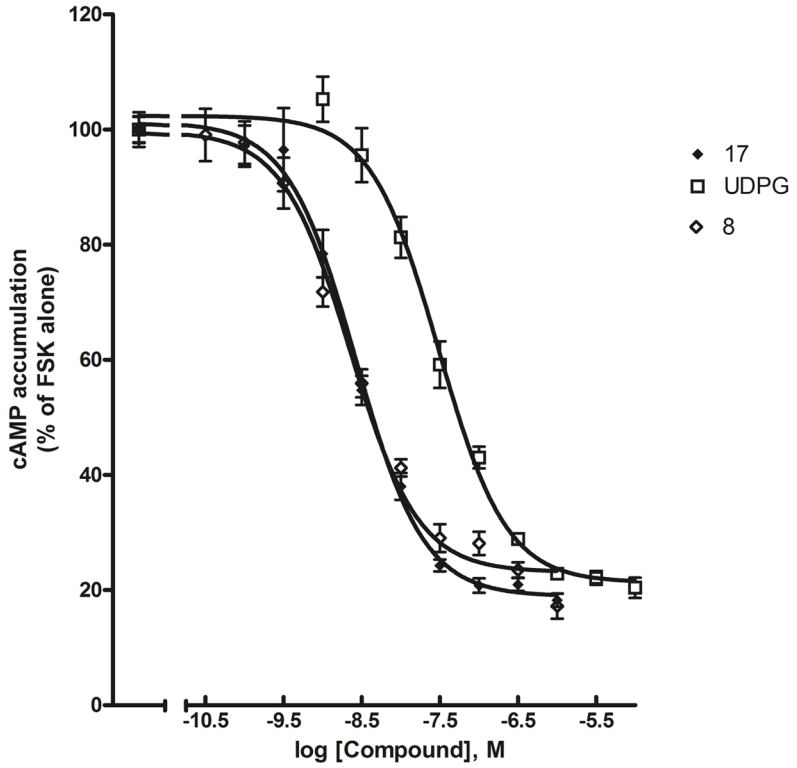


Figure 4.

Activation of the G_i-coupled $P2Y_{14}$ receptor stably expressed in C6 cells by the mixed nucleoside/nucleotide G4 dendrimer conjugate **8** (MRS5259) and the nucleotide G4 dendrimer conjugate **17** (MRS2949). Inhibition of forskolin (FSK)-stimulated cAMP is shown in comparison to the effects of UDPG (n = 3).

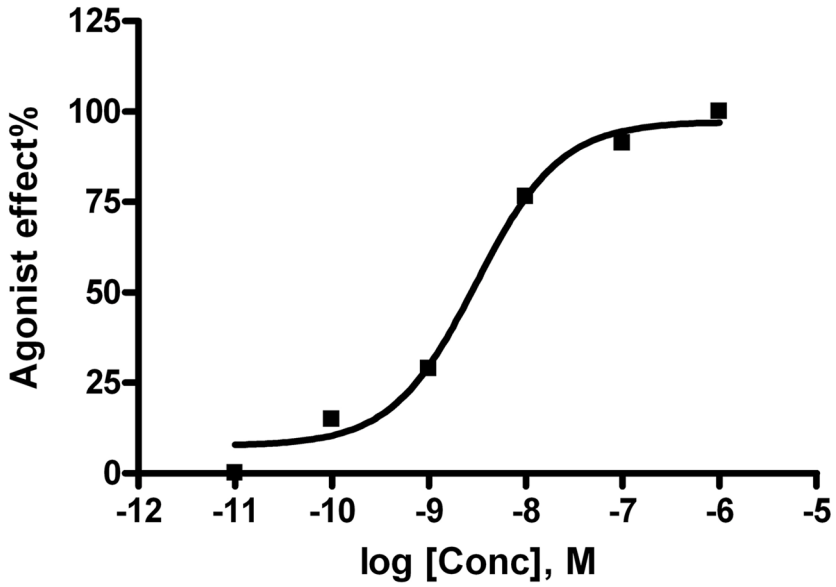
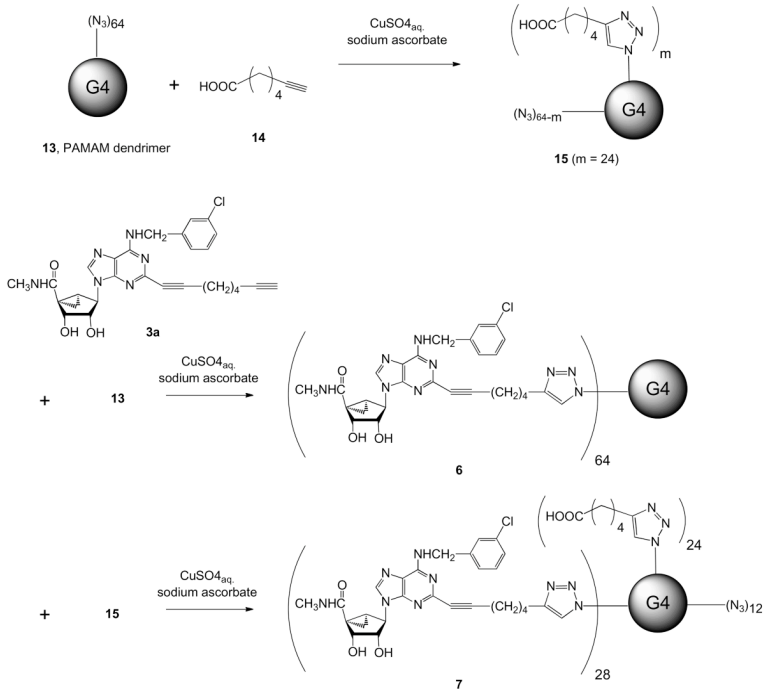


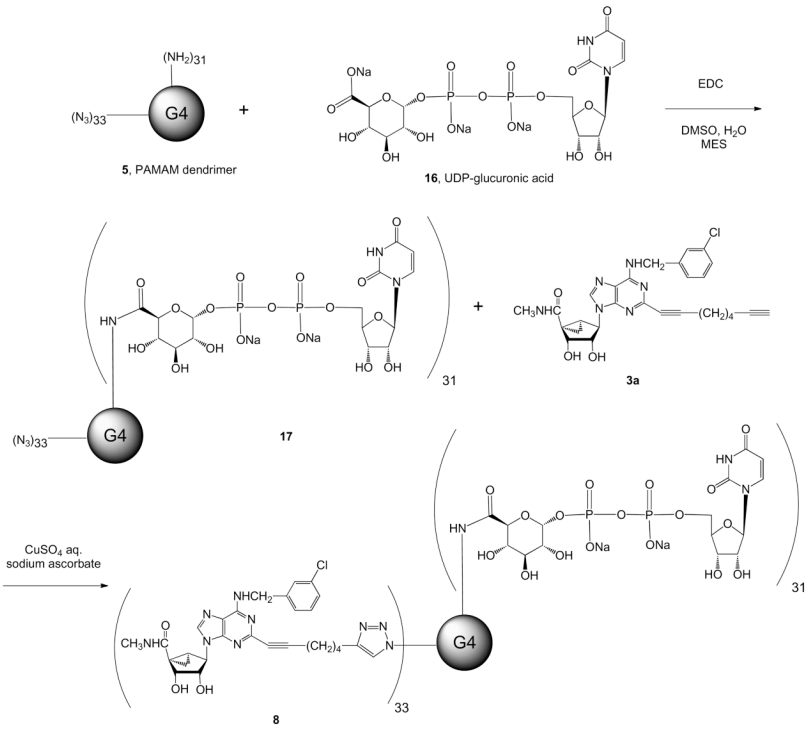
Figure 5.

Activation of the G_i-coupled A_3AR stably expressed in CHO cells by **11**. The agonist effect measured is inhibition of forskolin-stimulated cAMP production, in comparison to the maximal effect of a full agonist NECA at 1 μM. Curve is representative of three determinations.



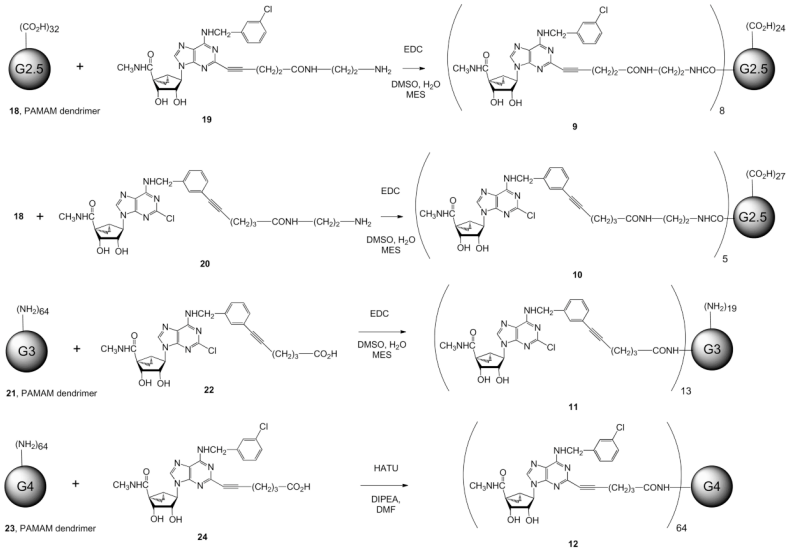
Scheme 1.

Use of click chemisry to synthesize PAMAM dendrimer derivatives **6** and **7**, containing a functionalized 2-alkynyl A_3AR agonist. An optional water-solubilizing group was added in **7**, in the form of an aliphatic carboxylic acid.

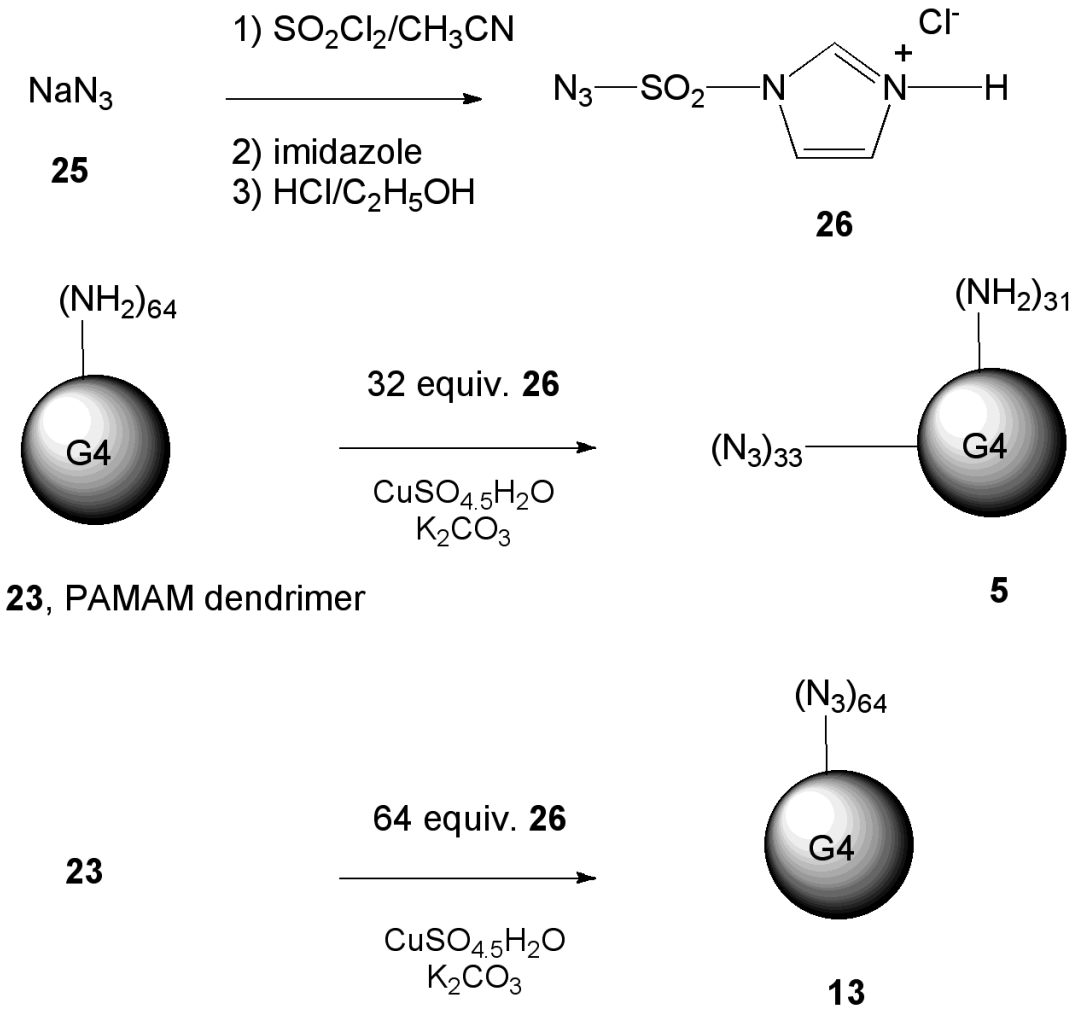


Scheme 2.

Synthesis of two PAMAM dendrimer derivatives containing a functionalized agonist of the $P2Y_{14}$ receptor derived by amide linkage of UDPGA **16**. Conjugate **17** contains only the $P2Y_{14}$ receptor agonist; conjugate **8** contains an A_3AR agonist linked through a click reaction of its 2-alkynyl functionalized chain. The dendrimer precursor **5** contained a random distribution of peripheral amino and azido groups in a ratio of ~33:31.



Scheme 3.
Preparation of amide-linked nucleoside conjugates of PAMAM dendrimers.



Scheme 4.

Synthesis of azido-derivatized PAMAM dendrimers, which served as precursors for the GPCR ligand conjugates by reacting with imidazole-1-sulfonyl azide hydrochloride **26**.

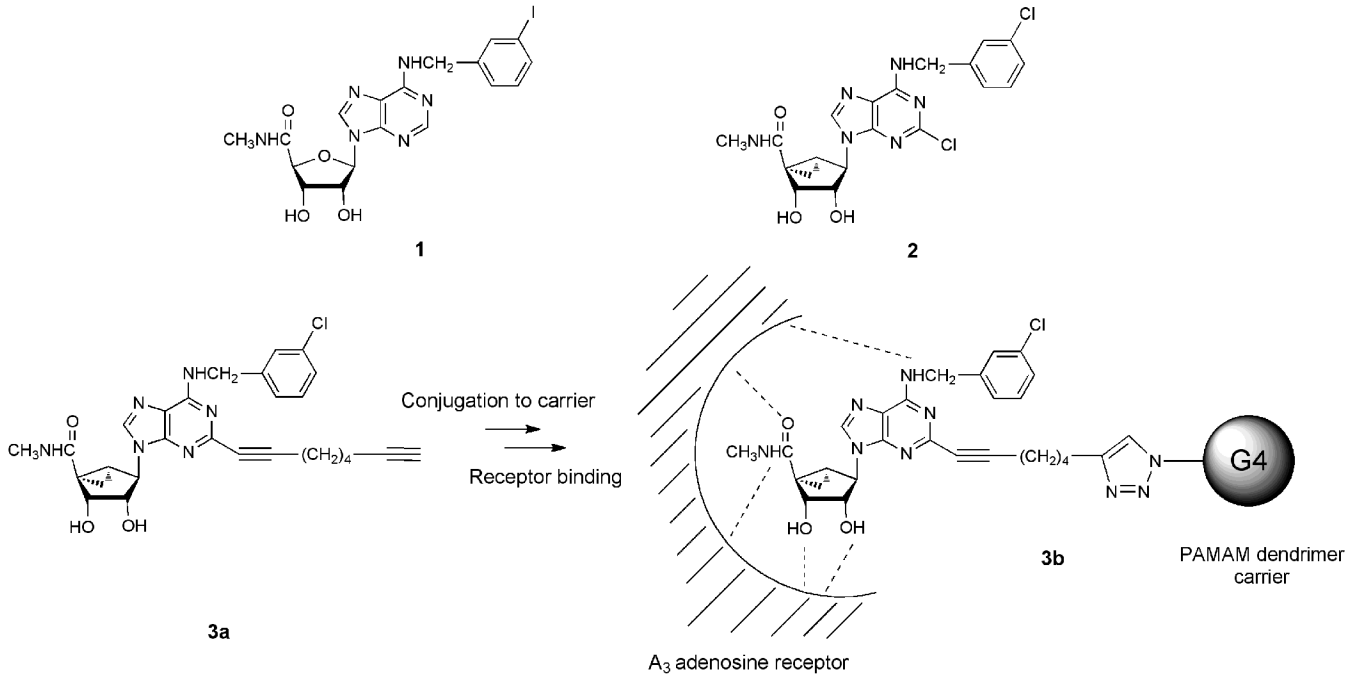
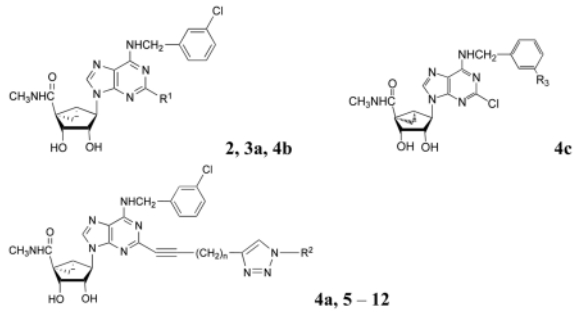


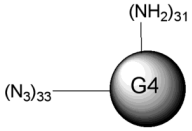
Chart 1.

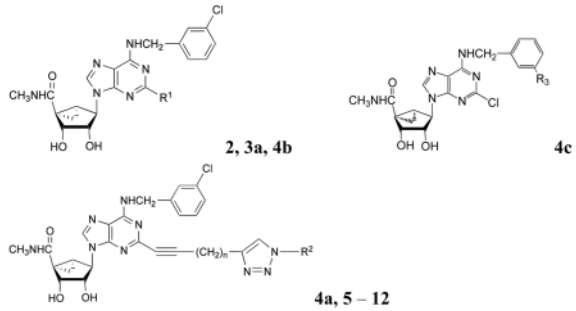
Structures of adenosine A_3AR agonist monomers (1–3) and the design of multivalent dendrimer conjugates in the(N) -methanocarba series, represented by the triazole derivative of general formula (4). Compound **3a** was reported in ref. [9].

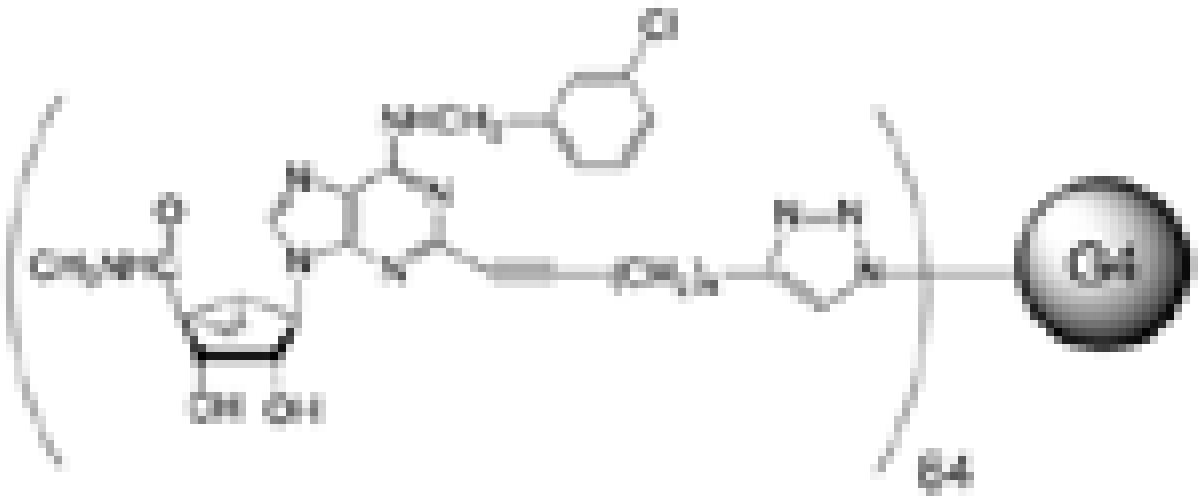
Table 1

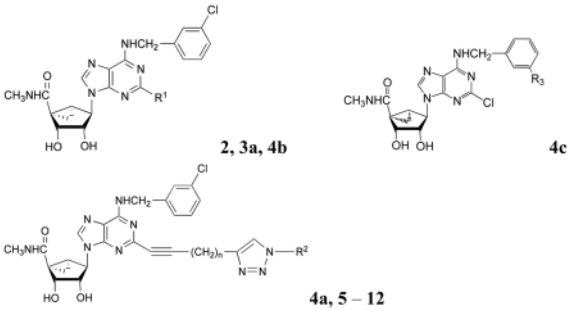
Potency of a series of nucleoside-dendrimer conjugates at three subtypes of human ARs.

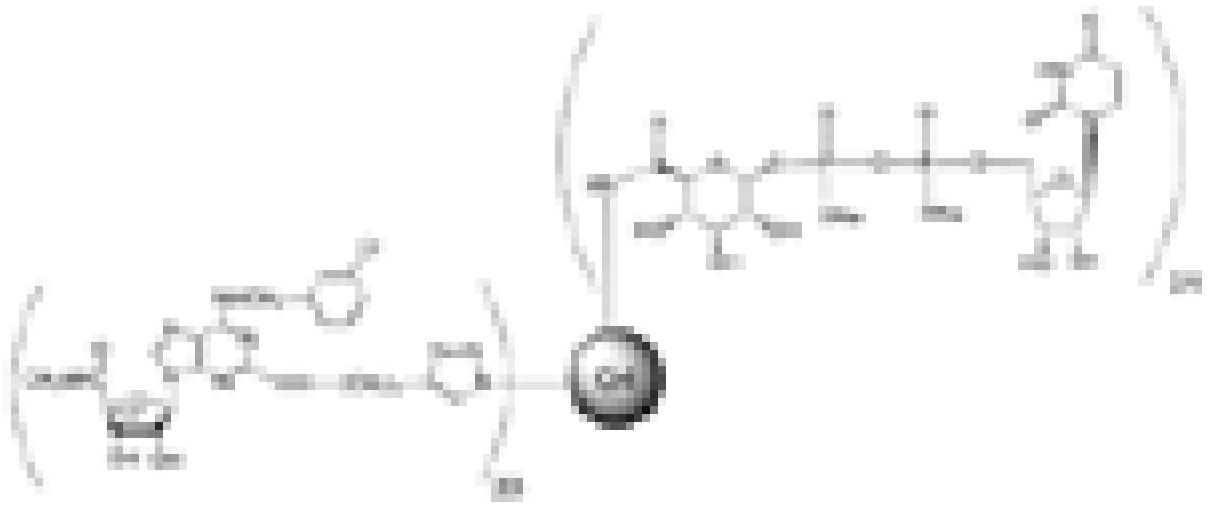
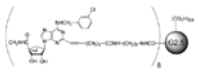
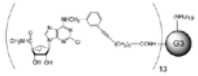


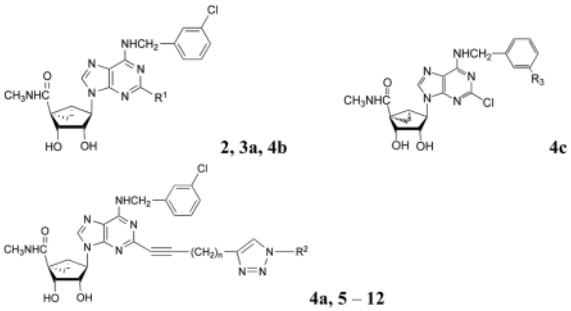
Compd	Structure, R^1, R^2, or R^3	Affinity A_1
Model compounds		
1[b] ***IB-MECA***		51
2[b,c]	R^1 = Cl	260±60[b]
3a[b,c]	R^1 = C≡C$(CH_2)_4$C≡CH	*(31±2%* at 10 μM
4a[b,c]	R^2 = $(CH_2)_2$NHCOCH_3	*(12±4%* at 10 μM
4b[b]	R^1 = C≡C$(CH_2)_2CH_3$	1040±80
4c[b]	R^3 = C≡C$(CH_2)_4$CONH$(CH_2)_2$NHCOCH_3	181±22
Dendrimer derivatives		
5[d]		NA

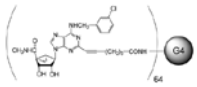


Compd	Structure, R^1, R^2, or R^3	Affinity A_1
6[c]	 64	22.6±6.9
7[c]	 24 28	35.1±7.5



Compd	Structure, R^1, R^2, or R^3	Affinity A_1
8[C]	 31 33	78±31
9	 8	ND
10	 5	77
11[C]	 13	7.1



Compd	Structure, R^1, R^2, or R^3	Affinity A_1
12	 64	1.92±1.2

[a] All experiments were done on CHO or HEK293 (A_{2A} only) cells stably expressing one of four subtypes of human ARs. The binding affinity for A_1, A_{2A}, and A_3ARs was expressed as K_i values (n = 3–5) and was determined by using agonist radioligands ([^{3}H]NECA, [^{3}H]CGS21680, or [^{125}I]I-AB-MECA, respectively), unless noted. A percentage in parentheses refers to inhibition of radioligand binding at the indicated concentration. The concentrations of the dendrimer-ligand complexes were measured by the concentration of the dendrimer, not the ligand. Therefore, all binding K_i values of dendrimers are expressed as K_{iapp} values.

[b] Affinity values from Tosh et al. (15,21) and Melman et al. (14).

[c] **2**, MRS3558; **3a**, MRS5221; **4a**, MRS5233; **6**, MRS5246; **7**, MRS5247; **8**, MRS5259; **11**, MRS5216.

[d] Coumpound **5** is the dendrimer precursor of **8** and is similar to the dendrimer precursor of **6** and **7**.

NA – not active in inhibition of radioligand binding (<10%) at 1 μM.

Table 2

Maximal efficacy of (N)-methanocarba adenosine derivatives in a functional assay at the A_3AR.

Compound	Concentration (μM)	% Inhibition of cAMP accumulation[a] at the hA_3AR
IB-MECA	1.0	99±6
Cl-IB-MECA	1.0	97
NECA	1.0	100
3a	10	60.2±17.0
4a[b]	10	93.6±17.7
5	1.0	1.7±1.4
6	0.1	98.7±14.3
7	0.1	91.4±20.2
8	0.1	111±20
9[c]	1.0	88.2±15.1
10	1.0	91.3±12.3
11	1.0	101±10
12	0.1	92.1±21.2

[a] The efficacy at the human A3AR was determined by inhibition of forskolin-stimulated cyclic AMP production in AR-transfected CHO cells, as described in the text. Percent inhibition is shown at the indicated concentration, in comparison to the maximal effect of a full agonist NECA at 1 μM. Data are expressed as mean ± standard error (n = 3–5). ND, not determined.

[b] Values from Tosh et al. (21).

[c] EC_{50} values were determined with full curves for compounds **7** (0.12 ± 0.03 nM), **8** (1.36 nM), and **11** (2.93 ± 0.37 nM).