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GPCR Ligand Dendrimer (GLiDe) Conjugates: Adenosine Receptor Interactions of a Series of Multivalent Xanthine Antagonists

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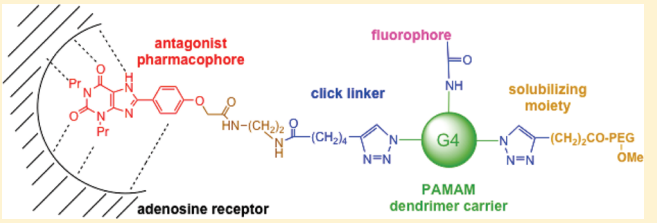
Supporting Information

ABSTRACT: Previously, G protein-coupled receptor (GPCR) agonists were tethered from polyamidoamine (PAMAM) dendrimers to provide high receptor affinity and selectivity. Here, we prepared GPCR ligand−dendrimer (GLiDe) conjugates from a potent adenosine receptor (AR) antagonist; such agents are of interest for treating Parkinson's disease, asthma, and other conditions. Xanthine amine congener (XAC) was appended with an alkyne group on an extended C8 substituent for coupling by Cu(I)-catalyzed click chemistry to azide-derivatized G4 (fourth-generation) PAMAM dendrimers to form triazoles. These conjugates also contained triazole-linked PEG groups (8 or 22 moieties per 64 terminal positions) for increasing water-solubility and optionally prosthetic groups for spectroscopic characterization and affinity labeling. Human AR binding affinity increased progressively with the degree of xanthine substitution to reach K_i values in the nanomolar range. The order of affinity of each conjugate was $hA_{2A}AR > hA_3AR > hA_1AR$, while the corresponding monomer was ranked $hA_{2A}AR > hA_1AR \geq hA_3AR$. The antagonist activity of the most potent conjugate **14** (34 xanthines per dendrimer) was examined at the G_i-coupled A_1AR. Conjugate **14** at 100 nM right-shifted the AR agonist concentration−response curve in a cyclic AMP functional assay in a parallel manner, but at 10 nM (lower than its K_i value), it significantly suppressed the maximal agonist effect in calcium mobilization. This is the first systematic probing of a potent AR antagonist tethered on a dendrimer and its activity as a function of variable loading.



INTRODUCTION

We have used poly(amidoamine) (PAMAM) dendrimers as polymeric nanocarriers for drugs that bind to G protein-coupled receptors (GPCRs) located on the cell surface.[1−3] These receptors are an important area of drug discovery. Agonists and antagonists of rhodopsin-like GPCRs constitute a large fraction (~30%) of the 324 molecular targets of FDA-approved drugs.[4] Multivalent GPCR ligand−dendrimer (GLiDe) conjugates, in which a strategically derivatized agonist or antagonist is covalently tethered from a high molecular weight PAMAM dendrimer, have displayed enhanced pharmacodynamic properties in comparison to those of the monomeric ligands.[2,3,5] One of the objectives of this approach is to favorably alter the pharmacokinetics of the drug in vivo when bound to the nanocarrier. It is also feasible to target the binding of a polymeric drug to aggregates of GPCRs, which have been shown to exist and have been postulated to be major participants in the varied biological effects of a given receptor.[6] GPCR dimers, including heterodimers of two different receptor classes, have already been established to have an altered pharmacology compared to that of homomeric GPCRs.[7] We have shown with molecular modeling that GLiDe conjugates of sufficient molecular size can adopt a suitable geometry to bridge multiple binding sites in such receptor aggregates.[8]

We initially reported the use of PAMAM dendrimers as scaffolds for the presentation of nucleosides and nucleotides that selectively activate adenosine receptors (ARs, particularly the A_{2A} and A_3 subtypes) or antagonize P2Y receptors (specifically the $P2Y_1$ subtype) to modulate intracellular signaling,[1,2,9] with both of these receptor families being GPCRs. These GLiDe conjugates are stable and biologically active without requiring the release of small molecules or cellular internalization, which are often an integral part of other polymeric drug delivery schemes.[10] Recently, we showed that a potent A_3AR agonist conjugate displayed cytoprotective effects in a cardiomyocyte culture heterologously expressing the A_3AR.[11] The present study extends the GLiDe conjugate approach for the first time from AR agonists to multivalent conjugates of AR antagonists. AR antagonists, the naturally occurring xanthines theophylline **1** and caffeine **2** being prototypical examples (Figure 1), are of therapeutic interest in the treatment of Parkinson's disease, diabetes, asthma, cancer, and other conditions.[4,12] The structure−activity relationship (SAR) of xanthine derivatives as AR antagonists has been exhaustively explored.[13,14] One of the earliest high affinity AR ligand probes was the 8-phenyl derivative xanthine amine congener (**3**, XAC), which is a moderately selective antagonist of the A_1AR subtype in the rat and is relatively nonselective in binding to human (h) ARs.[15] XAC contains an amine-terminal chain placed at an insensitive site on the pharmacophore and was designed as a functionalized

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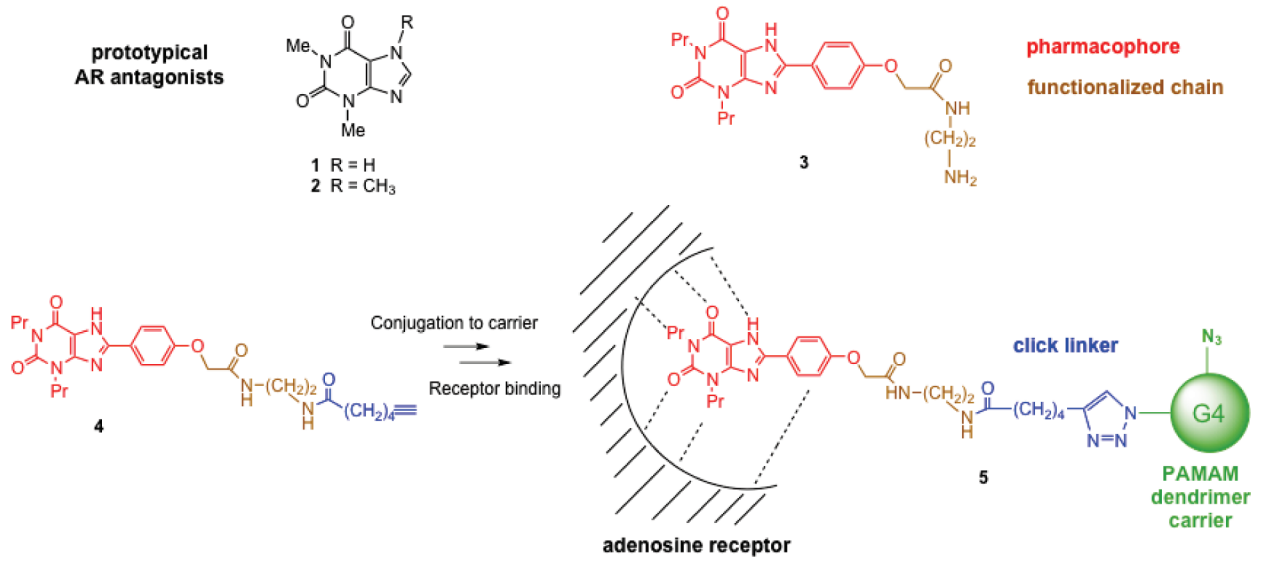


Figure 1. Structures of nonselective AR antagonists (**1**−**3**) and the design of a series of multivalent dendrimer conjugates of a potent xanthine, represented by the triazole derivative of unspecified stoichiometry in the general formula (**5**). Compound **3** is a functionalized XAC congener designed for attachment to carrier moieties with retention of the ability to bind potently to ARs. The conjugation of alkyne derivative **4** is performed using click chemistry, which forms a triazole linker on an insensitive site on the xanthine with respect to receptor recognition.

congener,[16] i.e., for attachment to larger carrier moieties and reporter groups with retention of the ability to bind potently to ARs.

In this study, we have identified a means of linking XAC to water-soluble PAMAM dendrimers by Cu(I)-catalyzed click chemistry between an alkyne and an azide,[17] which we found to preserve the high receptor affinity in this series. The degree of substitution by XAC and by polyethylene glycol (PEG) water-solubilizing chains was systematically varied and the effects on receptor affinity studied. This analysis suggests that each GLiDe conjugate molecule may bind to multiple AR binding sites in the vicinity on the cell surface. Furthermore, the ability to introduce prosthetic groups for receptor localization or characterization on the carrier without interfering with the high affinity in receptor binding was shown.

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) and the G4 PAMAM dendrimer (5 wt % solution in methanol) with an ethylenediamine core **6a** 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). Imidazole-1-sulfonyl azide hydrochloride was prepared.[18] Synthesis of XAC **3** is described elsewhere.[14] Active esters IRDye800CW **23** and IRDye700DX **24** were obtained from LI-COR Biosciences (Lincoln, NE). Amicon centrifugal ultrafilters (molecular weight cutoff 3000) were used.

Proton nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-400 spectrometer with d_6-DMSO as a solvent. The chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (δ 0.0 ppm) or in D_2O relative to HOD (4.80 ppm). ESI−high resolution mass spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine 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. 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. IR spectra were recorded using a PerkinElmer Spectrum One FT-IR spectrometer (applied as a DMSO solution).

*Azido-Derivatized G4 PAMAM dendrimer (**6b**).* The G4 PAMAM dendrimer containing an ethylene diamine core was obtained from Sigma-Aldrich and terminal amines reacted with imidazole-1-sulfonyl azide hydrochloride.[3] K_2CO_3 (3.48 g, 30.15 mmol), $CuSO_4 \cdot 5H_2O$ (34.6 mg, 0.163 mmol), and imidazole-1-sulfonyl azide hydrochloride (3.0 g, 1.04 mol) were added to a solution of G4 PAMAM dendrimer **6a**, (20 g, 10 w% in methanol, $[NH_2]$ = 16.3 mmol) in anhydrous methanol (11 mL), and the reaction mixture was stirred at room temperature overnight. The mixture was then dialyzed in deionized water for 7 days (changing water 4 times per day). Lyophilization after dialysis provided the azide-derivatized G4 dendrimer **6b** (3.2 g, 58%) as a solid. IR ν_{max} 2110 cm^{-1}. ESI-MS: calcd, 15,611; found, 15,545.

*G4 PAMAM, Conjugated with PEG [8] (**8**).* The azide-derivatized G4 PAMAM dendrimer **6b** (111 mg, 7.1 μmol) and a poly(ethylene glycol)methyl ether derivative of butynoic acid **7** (MW 2000, Aldrich Chemical Co., 114 mg, 57 μmol) were added to a mixture of DMSO (4 mL) and water (4 mL). The solution was treated with freshly prepared aqueous sodium ascorbate (1 M solution, 228 μL), followed by the addition of 7.5% aqueous cupric sulfate (380 μL, 113 μmol). The reaction mixture was stirred at room temperature (rt) overnight, and the product was purified by extensive dialysis in water (changing water 4 times per day for 2 days). The mixture was lyophilized to give compound **8**

(153 mg, 67%) containing ~8 equivalents of PEG per dendrimer molecule as a colorless foamy solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.10 (br s), 4.35 (s), 4.13 (s), 3.5 (s), 3.41−3.44 (m), 3.33 (br s), 3.24 (s), 2.67−2.87 (m), 2.26 (br s). ESI-MS: calcd, 31,545; found, 32,077.

*G4 PAMAM, Conjugated with PEG [22] (**9**).* Compound **9** (74%) containing ~22 equivalents of PEG per dendrimer molecule was synthesized from dendrimer **6b** following the same method as that for compound **8**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.03 (br s), 4.35 (s), 4.25 (s), 3.59 (s), 3.39−3.44 (m), 3.32 (br s), 3.24 (s), 2.87 (br s), 2.66 (br s), 2.33 (s), 2.24 (br s). ESI-MS: calcd, 59,545; found, 59,855.

*N-(2-(2-(4-(2,6-Dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)phenoxy)-acetamido)ethyl)hept-6-ynamide (**4**).* 6-Heptynoic acid (33 μL, 0.27 mmol) and EDC (68.8 mg, 0.36 mmol) were added to a solution of compound **3** (77 mg, 0.18 mmol) in anhydrous DMF (3 mL) and stirred overnight at rt. Solvent was evaporated, and the residue was purified using flash silica gel column chromatography to give compound **4** (69 mg, 72%) as a colorless solid. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.05 (d, J = 8.8 Hz, 2H), 7.14 (d, J = 8.8 Hz, 2H), 4.61−4.63 (m, 2H), 4.14 (t, J = 7.2 Hz, 2H), 4.01 (t, J = 7.6 Hz, 2H), 3.41−3.65 (m, 6H), 2.19−2.13 (m, 5H), 1.86−1.70 (m, 1H), 1.69−1.64 (m, 4H), 1.52−1.46 (m, 1H), 1.15 (d, J = 6.4 Hz, 1H), 1.17−0.95 (m, 6H). HRMS calculated for $C_{28}H_{37}N_6O_5$ (M + H) $^+$: 537.2825; found, 537.2823.

*G4 PAMAM, Conjugated with PEG [22] and the XAC [6] Derivative (**10**).* The azide-derivatized G4 PAMAM dendrimer **6b** (10.5 mg, 0.17 μmol) and a XAC alkyne derivative **4** (0.54 mg, 1.02 μmol) were added to a mixture of DMSO (0.4 mL) and water (0.4 mL). The solution was treated with freshly prepared aqueous sodium ascorbate (1 M solution, 3 μL), followed by the addition of 7.5% aqueous cupric sulfate (2.3 μL, 0.7 μmol). The reaction mixture was stirred at rt overnight, and the product was purified by extensive dialysis in water (changing water 4 times per day for 2 days). The mixture was lyophilized to give compound **10** (6.9 mg, 66%) containing ~22 equivalents of PEG and ~6 xanthine equivalents per dendrimer molecule as a foamy solid. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.15 (br s), 8.09 (d, J = 8.8 Hz), 7.71−7.97 (m), 7.16 (d, J = 9.2 Hz), 4.54 (s), 4.24−4.36 (m), 4.12 (br s), 4.04 (t, J = 7.6 Hz), 3.81 (t, J = 7.6 Hz), 3.54−3.67 (m), 3.49 (br s), 3.14−3.47 (br m), 2.63−2.71 (br m), 2.07−2.33 (m), 1.42−1.81 (br m), 0.84−0.92 (br m). ESI-MS: calcd, 63,074; found, 63,069.

*G4 PAMAM, Conjugated with PEG [22] and the XAC [8] Derivative (**11**).* Compound **11** (70%) was synthesized from PEG conjugated dendrimer **9** following the same procedure as that for compound **10** using 8 equivalents of the XAC alkyne derivative **4**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.13 (br s), 8.10 (d, J = 8.8 Hz), 7.87−7.93 (m), 7.11 (d, J = 9.2 Hz), 4.55 (s), 4.32−4.36 (m), 4.12 (br s), 4.04 (t, J = 7.6 Hz), 3.85 (t, J = 7.6 Hz), 3.59−3.67 (m), 3.46 (br s), 3.14−3.42 (br m), 2.67−2.86 (br m), 2.05−2.33 (m), 1.51−1.75 (br m), 0.86−0.91 (br m). ESI-MS: calcd, 64,147; found, 64,251.

*G4 PAMAM, Conjugated with PEG [22] and the XAC [19] Derivative (**12**).* Compound **12** (75%) was synthesized from PEG conjugated dendrimer **9** following the same procedure as that for compound **10** using 19 equivalents of the XAC alkyne derivative **4**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.17 (br s), 8.07 (d, J = 8.8 Hz), 7.69−7.93 (m), 7.11 (d, J = 9.2 Hz), 4.54 (s), 4.30−4.35 (m), 4.13 (br s), 4.02 (t, J = 7.6 Hz), 3.84 (t, J = 7.6 Hz), 3.58−3.68 (m), 3.51 (br s), 3.13−3.48 (br m), 2.62−2.7 (br m), 2.05−2.33 (m), 1.39−1.81 (br m), 0.86−0.92 (br m). ESI-MS: calcd, 70,050; found, 70,074.

*G4 PAMAM, conjugated with PEG [22] and XAC [23] derivative (**13**).* Compound **13** (78%) was synthesized from PEG conjugated dendrimer **9** following the same procedure as that for compound **10** using 23 equivalents of the XAC alkyne derivative **4**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.13 (br s), 8.09 (d, J = 8.8 Hz), 7.91−7.86 (m), 7.11 (d, J = 9.2 Hz), 4.54 (s), 4.32−4.34 (m), 4.12 (br s), 4.04 (t, J = 7.6 Hz), 3.86 (t, J = 7.6 Hz), 3.58−3.68 (m), 3.44 (br s), 3.14−3.44 (br m), 2.67−2.87 (br m), 2.03−2.33 (m), 1.49−1.75 (br m), 0.86−0.92 (br m). ESI-MS: calcd, 72,197; found, 72,320.

*G4 PAMAM, Conjugated with PEG [22] and the XAC [34] Derivative (**14**).* Compound **14** (84%) was synthesized from PEG conjugated dendrimer **9** following the same method as that for compound **10** using 34 equivalents of the XAC alkyne derivative **4**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.11 (br s), 8.09 (d, J = 9.2 Hz), 7.90−7.85 (m), 7.11 (d, J = 9.2 Hz), 4.54 (s), 4.02 (t, J = 6.0 Hz), 3.87 (t, J = 7.6 Hz), 3.12−3.71 (br m), 2.67−2.74 (m), 2.30−2.38 (m), 2.10−2.14 (m), 2.05 (t, J = 7.2 Hz), 1.72−1.79 (m), 1.53−1.61 (m), 1.37−1.41 (m), 0.86−0.92 (br m). ESI-MS: calcd, 78,100; found, 77,999.

*G4 PAMAM, Conjugated with PEG [8] and the XAC [4] Derivative (**15**).* Compound **15** (67%) was synthesized from PEG conjugated dendrimer **8** following the same method as that for compound **10** using 4 equivalents of the XAC alkyne derivative **4**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.14 (br s), 8.08 (d, J = 8.8 Hz), 7.72−7.90 (m), 7.10 (d, J = 8.8 Hz), 4.54 (s), 4.34 (br s), 4.13 (br s), 4.02 (t, J = 7.6 Hz), 3.86 (t, J = 7.5 Hz), 3.60−3.67 (m), 3.49 (br s), 3.08−3.44 (br m), 2.67−2.87 (br m), 2.22−2.33 (br m), 1.48−1.68 (br m), 0.86−0.92 (br m). ESI-MS: calcd, 34,223; found, 34,377.

*G4 PAMAM, Conjugated with PEG [8] and the XAC [16] Derivative (**16**).* Compound **16** (72%) was synthesized from PEG conjugated dendrimer **8** following the same method as that for compound **10** using 17 equivalents of the XAC alkyne derivative **4**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.15 (br s), 8.09 (d, J = 8.8 Hz), 7.81−7.86 (m), 7.11 (d, J = 8.8 Hz), 4.54 (s), 4.34 (s), 4.19 (t, J = 7.5 Hz), 4.04 (t, J = 7.6 Hz), 3.52 (br s), 3.14−3.44 (br m), 2.62−2.74 (m), 2.03−2.33 (m), 1.39−1.75 (br m), 0.89−0.92 (br m). ESI-MS: calcd, 40,662; found, 40,461.

*G4 PAMAM, Conjugated with PEG [8] and the XAC [25] Derivative (**17**).* Compound **17** (80%) was synthesized from PEG conjugated dendrimer **8** following the same method as that for compound **10** using 26 equivalents of the XAC alkyne derivative **4**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.16 (br s), 8.09 (d, J = 8.8 Hz), 7.86−7.88 (m), 7.11 (d, J = 8.8 Hz), 4.54 (s), 4.02 (t, J = 6.8 Hz), 3.89 (t, J = 6.7 Hz), 3.51 (br s), 3.14−3.32 (m), 2.10−2.14 (m), 2.04 (t, J = 7.2 Hz), 1.72−1.77 (m), 1.51−1.61 (m), 1.35−1.43 (m), 0.81−0.92 (br m). ESI-MS: calcd, 45,492; found, 45,223.

*G4 PAMAM, Conjugated with PEG [8] and the XAC [37] Derivative (**18**).* Compound **18** (84%) was synthesized from PEG conjugated dendrimer **8** following the same method as that for compound **10** using 37 equivalents of the XAC alkyne derivative **4**. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.18 (br s), 8.08 (d, J = 9.2 Hz), 7.86−7.88 (m), 7.11 (d, J = 8.8 Hz), 4.54 (s), 4.02 (t, J = 6.0 Hz), 3.87 (t, J = 6.8 Hz), 3.12−3.62 (br m), 2.67−2.74 (m), 2.33 (br s), 2.10−2.13 (m), 2.05 (t, J = 6.8 Hz), 1.72−1.77 (m), 1.53−1.61 (m), 1.37−1.43 (m), 0.88−0.92 (br m). ESI-MS: calcd, 51,931; found, 52,035.

*G4 PAMAM, Conjugated with PEG [8], XAC [37], and Alexa Fluor 488 (**20**).* A solution of Alexa Fluor 488 alkyne **19** (0.5 mg, 0.64 μmol) in water (0.5 mL) and freshly prepared sodium ascorbate (1 M, 5 μL) were added to a solution of the XAC-dendrimer conjugate **18** (8.2 mg, 0.16 μmol) in DMSO (0.5 mL). Then, 7.5% aqueous cupric sulfate (6 μL, 113 μmol) was added to the reaction mixture, which was stirred overnight at rt. The dendrimer derivative was purified by extensive dialysis in water, and lyophilization of the resulting solution gave compound **19** (5.9 mg, 66%) as an orange colored solid. ESI-MS: calcd, 57,065; found, 57,079.

*4-(2-Hept-6-ynamidoethyl)benzene-1-sulfonyl Fluoride (**21**).* 6-Heptynoic acid (11 μL, 0.09 mmol), HATU (31 mg, 0.08 mmol), and diisopropyl ethylamine (14 μL, 0.08 mmol) were added to a solution of 4-(2-aminoethyl)benzene-1-sulfonyl fluoride (Aldrich, 15 mg, 0.06 mmol) in anhydrous DMF (1 mL). The reaction mixture was stirred overnight at rt. The solvent was evaporated, and the residue was purified using flash silica gel column chromatography (hexane/ethyl acetate = 2:1) to give compound **21** (15.2 mg, 78%) as a colorless solid. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.00 (d, J = 8.4 Hz, 2H), 7.60 (d, J = 8.0 Hz, 2H), 3.52−3.48 (m, 2H), 2.98 (t, J = 6.8 Hz, 2H), 2.32 (t, J = 7.2 Hz, 2H), 2.23−2.20 (m, 2H), 1.75−1.44 (m, 5H). HRMS calculated for $C_{15}H_{19}NO_3SF$ $(M + H)^+$: 312.1070; found, 312.1073.

*G4 PAMAM, Conjugated with PEG [8], XAC [37], Alexa Fluor 488, and the Benzene-sulfonyl Fluoride Derivative (**22**).* Benzene-sulfonyl fluoride alkyne derivative **21** (0.5 mg, 1.6 μmol) and freshly prepared sodium ascorbate (1 M, 3 μL) were added to a solution of XAC-dendrimer conjugate **20** (5.6 mg, 0.1 μmol) in DMSO (0.4 mL). Then, 7.5% aqueous cupric sulfate (4 μL, 0.9 μmol) was added to the reaction mixture, which was stirred overnight at rt. The dendrimer derivative was purified by extensive dialysis in water, and lyophilization of the resulting solution gave compound **22** (4.2 mg, 73%) as an orange colored solid. ESI-MS: calcd, 58,945; found, 59,155.

*G4 PAMAM, Conjugated with PEG [22], XAC [34], and the IR Dye 800 Derivative (**25**).* A solution of IR dye 800CW NHS ester **23** (0.5 mg, 0.42 μmol) in DMF (0.3 mL) was added to a solution of compound **14** (2.66 mg, 0.05 μmol) in DMF (1 mL) followed by triethylamine (10 μL, 0.42 μmol) and stirred overnight at rt. The dendrimer derivative was purified by extensive dialysis in water, and lyophilization of the resulting solution gave compound **25** (2.13 mg, 76%) as a green colored foamy solid. ESI-MS: calcd, 81,943; found, 82,258.

*G4 PAMAM, Conjugated with PEG [22], XAC [34], the IR Dye 700 Derivative (**26**).* A solution of IR dye 700DX NHS ester **24** (0.5 mg, 0.25 μmol) in DMF (0.3 mL) was added to a solution of compound **14** (2.32 mg, 0.03 μmol) in DMF (1 mL) followed by sodium tetraborate buffer (20 μL, 0.25 μmol), and the reaction mixture was stirred overnight at rt. The dendrimer derivative was purified by extensive dialysis in water, and lyophilization of the resulting solution gave compound **26** (2.19 mg, 83%) as a green colored foamy solid. ESI-MS: calcd, 88,511; found, 89,063.

Biological Methods. *Materials.* Penicillin−Streptomycin−Glutamine and Hygromycin B were purchased from Invitrogen (Carlsbad, CA). [^{3}H]*R*-*N*^{6}-(2-Phenylisopropyl)adenosine ([^{3}H]R-PIA, 42.6 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). [^{125}I]4-Amino-3-iodobenzyl-5′-*N*-methylcarboxamidoadenosine ([^{125}I]I-AB-MECA, 2200 Ci/mmol) and [^{3}*H*]-2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS21680, 40.5 Ci/mmol) were purchased from PerkinElmer (Waltham, MA). DMEM/F12 medium, 1 M Tris-HCl (pH 7.5), and G418 Sulfate were purchased from Mediatech, Inc. (Herndon, VA). The calcium assay kit was from Molecular Devices (Sunnyvale, CA). All other reagents were from standard sources and are of analytical grade.

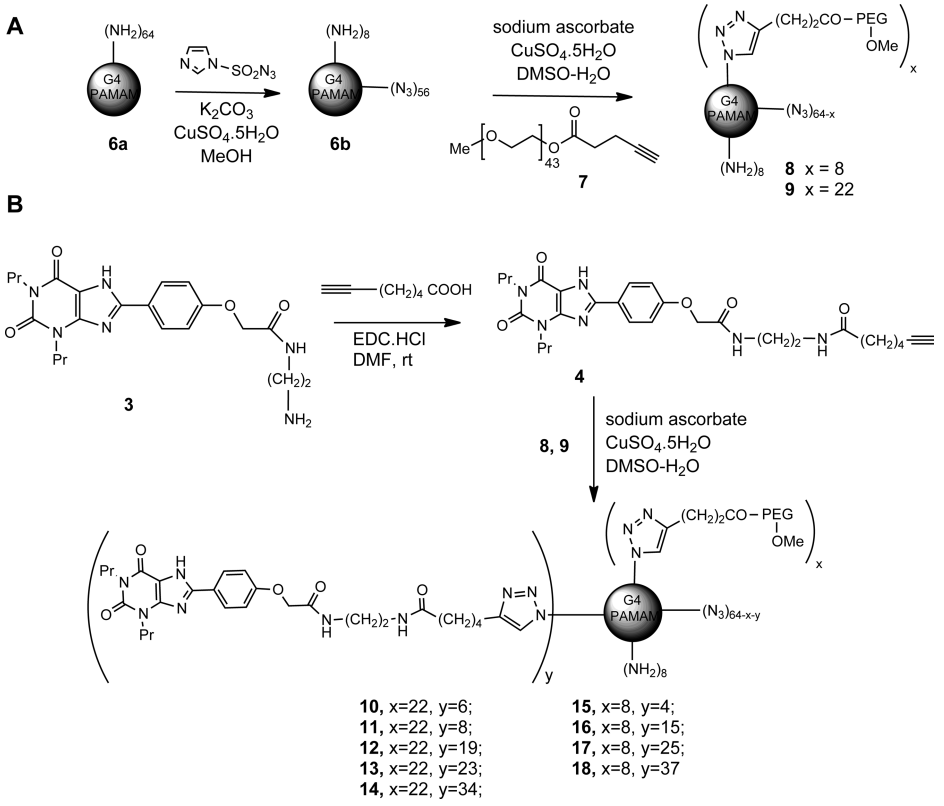
We prepared test compounds as 0.1 mM stock solutions in DMSO and stored them at 4 °C. Adenosine deaminase (25.3 U/mg) was purchased from Worthington Biochemical Corporation (Lakewood, NJ.)

Cell Culture and Membrane Preparation. We cultured Chinese hamster ovary (CHO) cells stably expressing the recombinant hA_1AR, $hA_{2B}AR$, and hA_3AR 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 (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin.[19] In addition, we added 800 μg/mL Geneticin to the $hA_{2A}AR$ media and 500 μg/mL Hygromycin B to the hA_1AR, $hA_{2B}AR$, and hA_3AR media. After harvesting the cells, we centrifuged them at 250*g* for 5 min at 4 °C. The pellet was resuspended in 50 mM Tris-HCL buffer (pH 7.5), containing 10 mM $MgCl_2$. The suspension was homogenized with an electric homogenizer for 10 s and was then recentrifuged at 20,000*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 then stored at −80 °C until the binding experiments were conducted. We measured the protein concentration with BCA Protein Assay Kit from Pierce Biotechnology (Rockford, IL).[20]

Binding Assays. Binding assays using standard AR ligands were performed using the general methods reported.[21−23] 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 of membrane suspension, added sequentially. For the hA_1AR (20 μg protein/tube), we used the radioligand [^{3}H]R-PIA (0.2 nM, precise final concentration is calculated for each experiment). For the $hA_{2A}AR$ (20 μg protein/tube), we used the radioligand [^{3}H]CGS21680 (10 nM). For the hA_3AR (20 μg protein/tube), we used the radioligand [^{125}I]I-AB-MECA (0.89 nM). We determined nonspecific binding with a final concentration of 10 μM unlabeled NECA diluted 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 reduced pressure with an M-24 cell harvester (Brandel, Gaithersburg, MD). We washed filters three times with 3 mL of 50 mM ice-cold Tris-HCl buffer (pH 7.5). We then placed filters for hA_1AR and $hA_{2A}AR$ binding in scintillation vials containing 5 mL of Hydrofluor scintillation buffer and counted with a PerkinElmer Liquid Scintillation Analyzer (Tri-Carb 2810TR). We counted filters for hA_3AR binding with a Packard Cobra II γ-counter (PerkinElmer).

Cyclic AMP Accumulation Assay. CHO cells expressing the A_1AR or $A_{2B}AR$ were seeded in 24-well plates and incubated at 37 °C overnight. The following day, the medium was removed and replaced with DMEM containing 50 mM HEPES, 10 μM rolipram, 3 U/mL adenosine deaminase, and increasing concentrations of agonists. For measurements at the A_1AR, forskolin (10 μM) was added 30 min after the addition of agonists and incubated for another 15 min. Antagonists were added 20 min before the addition of agonists. The medium was removed, and the cells were lysed with 200 μL of 0.1 M HCl. One hundred microliters of the HCl solution was used in the Sigma Direct

Scheme 1. Use of Click Chemistry in Sequential Steps to Synthesize PAMAM Dendrimer Derivatives 9−18, Containing a Functionalized AR Antagonist (Average Structures Shown)[a]



[a] (A) PEG moieties were added as water-solubilizing groups, followed by conjugation of the xanthine alkyne derivative **4** (B). Compound **7** was calculated to contain approximately 43 PEG groups on the basis of its molecular weight (2000 D).

cAMP Enzyme Immunoassay following the instructions provided with the kit. The results were interpreted using a BioTek ELx808 Ultra Microplate reader (BioTek, Winooski, VT) at 405 nm.

Intracellular Calcium Mobilization. CHO cells expressing the A_1AR cells were grown overnight in 100 μL of media in 96-well flat bottom plates at 37 °C at 5% CO_2 or until 80−90% confluency. The calcium assay kit (Molecular Devices, Sunnyvale, CA) was used as directed without washing cells and with probenecid added to the loading dye at a final concentration of 2.5 mM to increase dye retention. Cells were loaded with 50 μL of dye with probenecid to each well and incubated for 60 min at rt. The compound plate was prepared using dilutions of various compounds in Hanks Buffer (pH 7.4). Samples were run on a FLIPR^{TETRA} (Molecular Devices) in duplicate at rt. Antagonists were added 20 min before the addition of agonists. Cell fluorescence was measured (excitation at 485 nm; emission at 525 nm) following exposure to agonists. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure.

Data Analysis. We initially 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 the mean ± SE. The number of xanthine moieties on a given dendrimer that are accessible to receptors is uncertain; the data are therefore shown as apparent K_i values. Later, the same data were converted to the concentration of the xanthine moieties for graphic presentation. We repeated all experiments at least three times.

RESULTS

Chemical Synthesis. We prepared generation 4 (G4) PAMAM dendrimer conjugates of the potent, nonselective hAR antagonist XAC **3** (Scheme 1 and Table 1, **9**−**18**) to act as multivalent ligands of the receptors. The peripheral groups of the precursor dendrimer **6a** were modified to contain predominantly azido groups in place of the 64 terminal primary amines by reacting with imidazole-1-sulfonyl azide hydrochloride in methanol to give compound **6b**.[3,18] The same approach of preparing the PAMAM dendrimer for click chemistry by the substitution of amino groups with azides was taken in the synthesis of conjugates of AR agonists and $P2Y_1$ receptor antagonists.[3,9] Mass spectral data of **6b** indicated that 8 amino groups out of the 64 remained unreacted, and the presence of azido groups was confirmed by a strong IR peak at 2110 cm^{-1}. The presence of these residual amines was later used for synthetic advantage in the coupling of additional prosthetic groups. Prior to coupling of the pharmacophoric species, the dendrimer was appended with PEG moieties by a Cu(I)-catalyzed click cyclization using an alkyne-derivatized PEG **7** (end-capped by a methyl ether and a butynoyl ester) to yield the water-soluble and mainly uncharged PAMAM derivatives **8** and **9**, having 8 or 22 PEG moieties per dendrimer, respectively. Thus, two degrees of substitution with PEG of the 64-terminal groups of the G4 dendrimer were compared.

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Table 1. Binding Affinity of a Series of XAC Dendrimer Conjugates (10−22), Dendrimer Precursors (9, $(PEG)_{22}$ series; 8, $(PEG)_8$ series), and Small Molecular Monomers (3 and 4) at Three Subtypes of Human ARs[a]

compound	# of XAC moieties/dendrimer	$K_{i,app}$ or *% inhibition*, A_1AR	$K_{i,app}$ or *% inhibition*, $A_{2A}AR$	$K_{i,app}$ or *% inhibition*, A_3AR
3[b]	(monomer)	8.3 ± 1.3 nM	5.8 ± 2.3 nM	13.8 ± 4.4 nM
4[b]	(monomer)	107 ± 13 nM	23.2 ± 11.2 nM	175 ± 15 nM
9[c]	0	*21.8 ± 17.8%*	*29.7 ± 4.1%*	*70.2 ± 3.7%*
10	6	*34.3 ± 14.2%*	*45.9 ± 2.3%*	*71.7 ± 6.7%*
11	8	*59.2 ± 3.5%*	61.5 ± 23.0 nM	219 ± 67 nM
12	19	91.0 ± 15.6 nM	11.4 ± 5.0 nM	30.6 ± 3.8 nM
13	23	47.1 ± 3.4 nM	4.2 ± 1.7 nM	18.1 ± 5.2 nM
14[b,c]	34	15.0 ± 4 nM	2.6 ± 0.6 nM	8.9 ± 3.3 nM
8[c]	0	*10.3 ± 5.2%*	*4.4 ± 5.8%*	*51.9 ± 3.6%*
15	4	193 ± 117 nM	32.7 ± 6.0 nM	99.7 ± 25.0 nM
16	15	95.4 ± 35.3 nM	15.1 ± 1.7 nM	48.7 ± 20.2 nM
17	25	54.3 ± 17.1 nM	4.6 ± 0.4 nM	25.2 ± 3.8 nM
18[b,c]	37	29.0 ± 7.1 nM	4.2 ± 0.3 nM	14.5 ± 4.4 nM
20	37	20.2 ± 6.5 nM	4.1 ± 0.2 nM	6.2 ± 0.9 nM
22	37	29.3 ± 8.2 nM	5.4 ± 1.8 nM	7.8 ± 1.2 nM
25[b]	34	ND	ND	6.8 ± 2.1 nM
26[b]	34	ND	3.7	20.3 ± 16.4 nM

[a] All experiments were done on CHO (A_1 and A_3AR) or HEK293 ($A_{2A}AR$) 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]R-PIA, [^{3}H]CGS21680, or [^{125}I]I-AB-MECA, respectively), unless noted. The percentage inhibition refers to the inhibition of radioligand binding at the concentration of 1 μM. 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. ND: not determined. [b] **3**, XAC; **4**, MRS5398; **14**, MRS5359; **18**, MRS5385; **25**, MRS5421; **26**, MRS5422. [c] PEGylated dendrimer **9** is the precursor of **10**−**14**; PEGylated dendrimer **8** is the precursor of **15**−**18**. Xanthine-derivatized **18** is the dendrimer precursor of **20** and **22**. Xanthine-derivatized **14** is the dendrimer precursor of **25** and **26**.

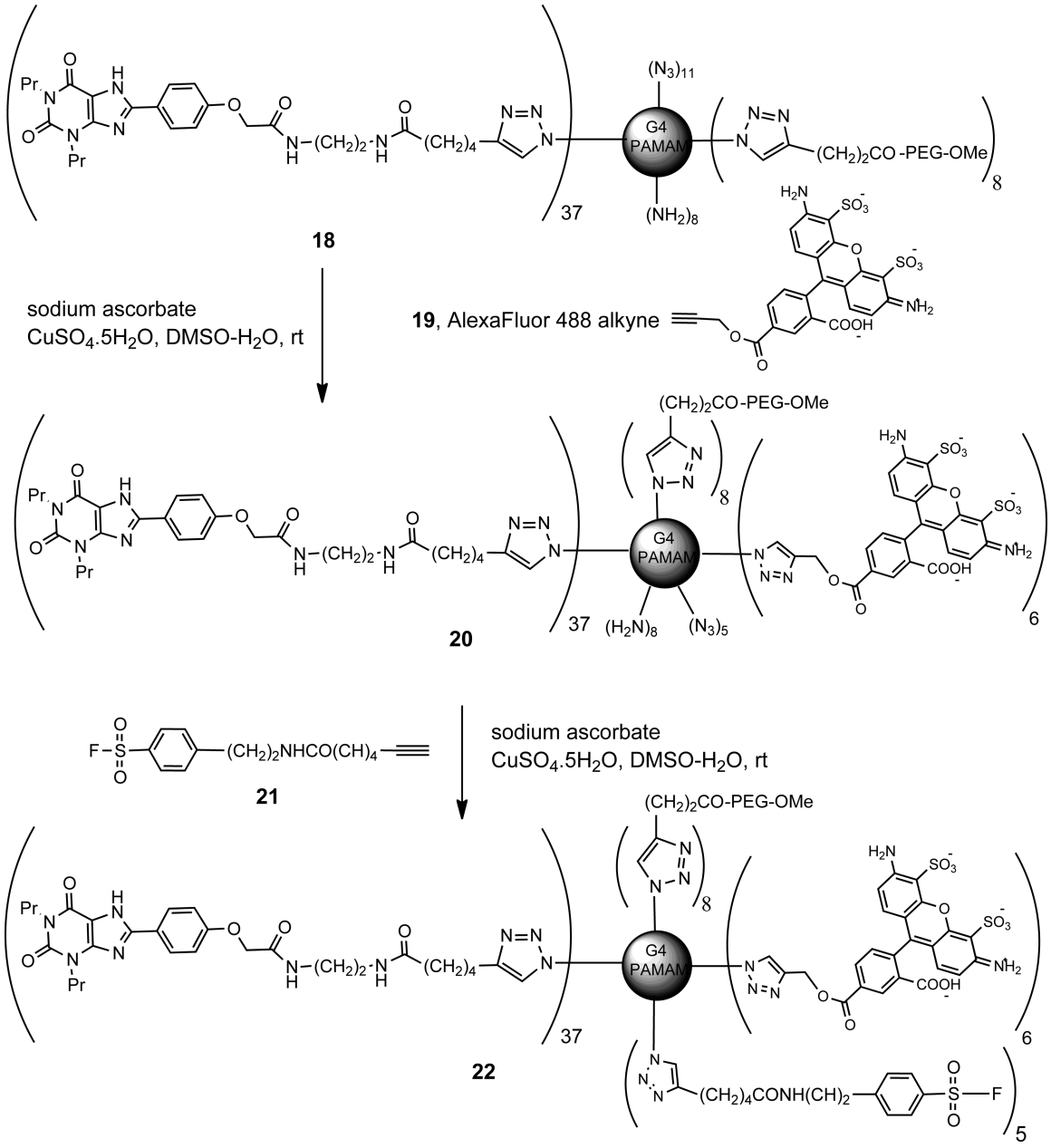
The synthetic method for cross-linking the receptor ligand to the nanocarriers **8** and **9** by click chemistry[3] required the introduction of an alkyne at an insensitive site on the xanthine moiety, and for this purpose, the hexynoyl amide derivative of XAC **4** was prepared. We have explored in detail the SAR at ARs at the distal region of the chain extended from the *para* position of the 8-phenylxanthines.[15,16] This is a site for unlimited chain derivatization, and thus, the alkyne-functionalized chain was used to provide click products with the azide-functionalized dendrimers.[3] We systematically varied the degree of substition of the dendrimer carrier with the GPCR ligand, i.e., the nonselective AR antagonist, ranging from substitution of an average of 4 to 37 of the terminal groups with the xanthine moiety. The efficiency of incorporation of the xanthine was nearly quantitative depending on the equivalents of **4** added to the click reaction, as indicated by mass spectroscopy. A fully XAC-substituted dendrimer was not prepared due to the limitation of aqueous solubility, which presented a concern in the absence of tethered PEG groups.

XAC conjugate **18**, which contained a random distribution of an average of 37 xanthine moieties and 8 PEG groups, was further derivatized with functional prosthetic groups that were coupled by click chemistry (Scheme 2). A fluorescent prosthetic group for spectroscopic characterization (Alexa Fluor 488) was included in conjugates **20** and **22**.[24] Alexa Fluor 488, which has emission and excitation maxima at 495 and 519 nm, respectively, has already been used as a fluorophore in small ligand probes of the $A_{2A}AR$.[25] Conjugate **22** also contained an aryl sulfonyl fluoride (an average of 5 moieties incorporated per PAMAM dendrimer) for affinity labeling of the receptor.[26] Aryl sulfonyl fluorides readily react irreversibly with proximal nucleophilic amino acids on a receptor yet are sufficiently stable to be applied in aqueous media. Two near-infrared (NIR) dyes[27] with absorption in the range of 800 nm (**25**) and 700 nm (**26**) were included in conjugates containing an average of 34 xanthine moieties per dendrimer. The NIR dyes were amide-linked to dendrimer **14** using the active ester precursors **23** and **24**, leading to an average degree of incorporation of 4 or 6 dye moieties incorporated per PAMAM dendrimer in **25** and **26**, respectively, as shown in Scheme 3.

Radioligand Binding. We tested the xanthine−dendrimer conjugates in binding assays at three hAR subtypes; we used standard radioligands[21−23] and membrane preparations from CHO cells (hA_1AR and hA_3AR) or HEK293 cells ($A_{2A}AR$) stably expressing a hAR subtype.[28,29] Binding affinity at the $A_{2B}AR$ was not determined because of the lack of a facile radioligand binding assay, but one representative conjugate was examined in a functional assay at this subtype.[19] Binding results shown in Table 1 indicated that receptor affinity was maintained in many of the conjugates. We used a previously reported monomeric antagonist of the ARs, XAC **3**,[16] and its hexynoyl derivative **4**, the synthetic precursor of the conjugates, for comparison in the binding assays. We included the parent dendrimers **8** and **9**, which contained a mixture of PEG, azido, and amino terminal groups, as controls; they were inactive or only weakly active in inhibiting the binding of hA_1AR and $hA_{2A}AR$ radioligands. The parent dendrimers **8** and **9** at 1 μM inhibited binding at the hA_3AR by 72% and 52%, respectively. However, we were not able to plot a full sigmoidal curve in the competition binding experiments to determine a K_i value. The relatively high displacement of radioligand at the hA_3AR by the parent dendrimers in high concentration may be related to the presence of aromatic triazolo rings on the surface of the dendrimer.

Inhibition of radioligand binding by dendrimer conjugates **14** and **18** in membranes of CHO cells expressing the hA_1 and A_3

Scheme 2. Synthesis of PAMAM Dendrimer Derivatives That Combined an Antagonist of the ARs and Functional Prosthetic Groups That Were Coupled by Click Chemistry (Average Structures Shown)[a]



[a] These consisted of conjugates for fluorescent detection (**20**) and for irreversible binding to the receptors (**22**). The dendrimer precursor **18** contained a random distribution of 37 xanthine moieties.

ARs and HEK-293 cells expressing the $hA_{2A}AR$ is shown in Figure 2B,C. Inhibition of radioligand binding by the xanthine monomer **4** is shown for comparison in Figure 2A. The results were also plotted graphically to illustrate the dependence of the affinity on the degree of substitution (Figure 3). Two methods of calculating the data were used: initially according to the concentration of the dendrimer, which is a single multivalent molecule as was done in previous studies,[1,2,5,9] or according to the concentration of the pendant xanthine moieties. The latter method is a mathematical construct, not an authentic equilibrium constant, because the xanthine moieties could not all be available to compete for receptor binding sites, by virtue of spatial limitations.

There was a smooth dependence of the affinity on the degree of substitution of the XAC-derivatized click-linked dendrimers in both series containing 8 or 22 PEG groups out of a total of 64 terminal sites. The more highly substituted dendrimer derivatives were considerably more potent than lightly substituted conjugates in binding assays at the hA_1AR, $hA_{2A}AR$, and hA_3AR. Thus, there was a regular progression toward increased affinity at each of these AR subtypes for **10**−**14** (from 6 to 34 xanthine moieties) in the more heavily PEGylated series and for **15**−**18** (from 4 to 37 xanthine moieties) in the more lightly PEGylated series. The dendrimer conjugates having 8 PEG groups displayed K_i values at the $hA_{2A}AR$ that varied from 33 nM to 4 nM ($n = 3$), for **15** and

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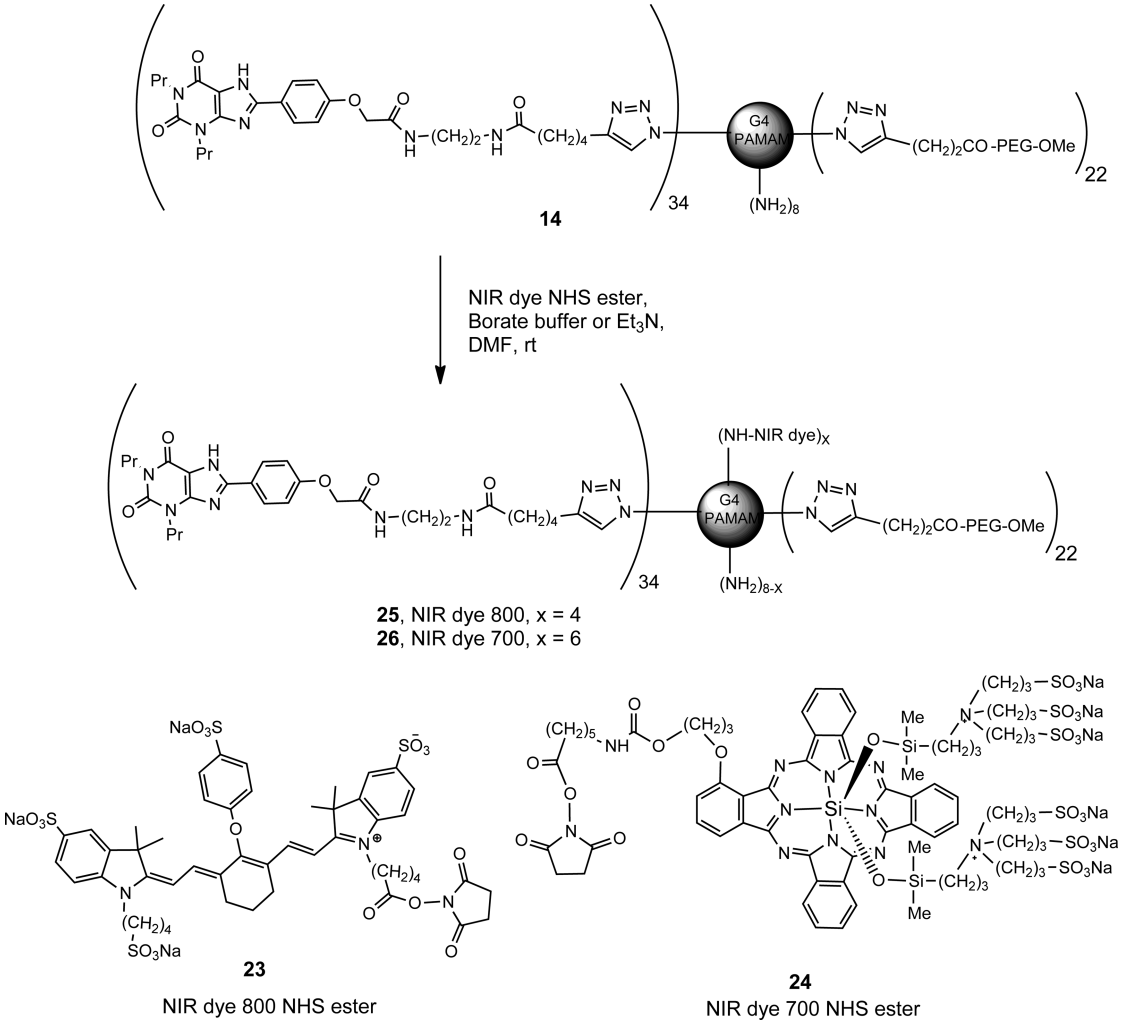
Scheme 3. Synthesis of PAMAM Dendrimer Conjugates That Combined an Antagonist of the ARs and Prosthetic Groups for NIR Detection That Were Coupled through Amide Linkage (Average Structures Shown)[a]



[a] The dendrimer precursor **14** contained a random distribution of 34 xanthine moieties.

18, respectively. The gain of affinity for the more highly PEGylated dendrimer series (Figure 3C and D) was steeper than the gain in the less highly PEGylated dendrimer series (Figure 3A and B). Thus, the affinity of the most highly substituted conjugate having 22 PEG groups (**14**) in binding to the ARs was particularly high, with K_i values of 15, 2.6, and 8.9 nM at hA_1AR, $hA_{2A}AR$, and hA_3AR, respectively. Therefore, the binding affinity at hAR subtypes (inversely related to apparent K_i) of these multivalent conjugates depended mainly on the degree of loading with the pharmacophore and secondarily on the degree of PEG substitution.

The order of affinity at AR subtypes for each conjugate was generally $hA_{2A}AR > hA_3AR > hA_1AR$. Therefore, a slight selectivity of these xanthine−dendrimer conjugates for the $hA_{2A}AR$ in comparison to that of the other ARs was demonstrated (3- to 6-fold for **14** and 4- to 7-fold for **18**). However, the corresponding monomers **3** and **4** displayed affinity in the order of $hA_{2A}AR > hA_1AR \geq hA_3AR$. Therefore, conjugation to the carrier altered the pharmacological profile of the xanthine by relatively increasing the A_3AR affinity. It is unknown whether this difference is due to the multivalency or to the chemical nature of the linker and its effect on interaction with the extracellular regions of the receptor.

The association of binding activity of the conjugate with a macromolecular species was confirmed by filtering a solution of compound **14** through a 3000 MW cutoff centrifugal filter. Both the UV absorption of the retained fraction and its binding activity were maintained following ultrafiltration. Thus, the observed activity was due to the intact conjugate, rather than a diffusable small molecular weight impurity.

High affinity in AR binding was maintained in the conjugates containing prosthetic groups for receptor characterization (**20**, **22**, **25**, and **26**). In fact, the K_i values of these conjugates were nearly the same as those of the precursors, i.e., XAC-conjugates **14** and **18**. This was a striking observation considering the structural diversity of the attached prosthetic groups. Compound **22** might have a component of nonequilibrium binding because of the chemically reactive sulfonyl fluoride group, but this was not evident from these binding results. The affinity of the 800 nm NIR dye in **25** was identical to precursor **14**, but the more sterically bulky NIR dye in **26** caused a slight (3-fold) loss of affinity at the A_3AR.

We further examined the A_1AR antagonist activity of compound **14**, one of the most potent conjugates for the AR subtypes

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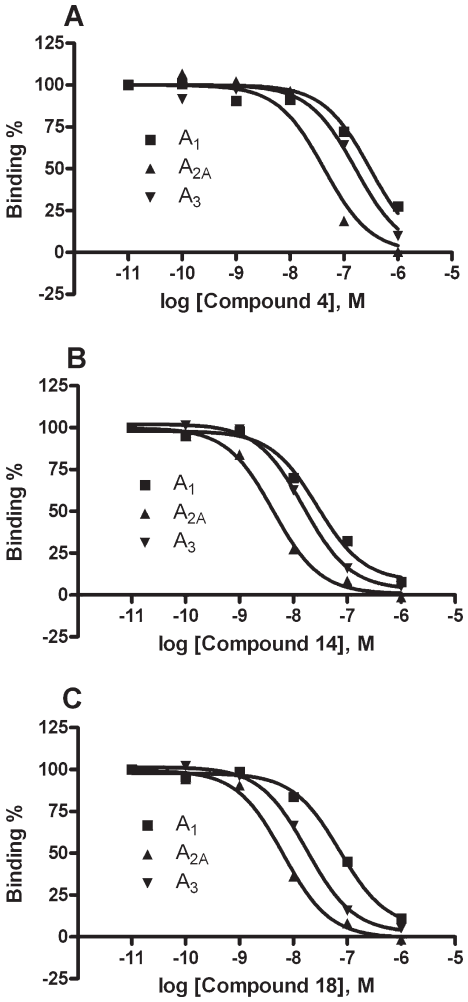

Figure 2. Inhibition of radioligand binding by xanthine monomer **4** (A) and dendrimer conjugates **14** (B) and **18** (C) (according to the concentration of the dendrimer) in membranes of CHO cells expressing the hA_1 and A_3 ARs and HEK-293 cells expressing the $hA_{2A}AR$.

in two types of functional assays in stably transfected CHO cells. We did not assay the functional effects at all the ARs; the G_i-coupled A_1AR is only representative of the full biological spectrum of the xanthines, and the behavior of the conjugate could be different at each of the AR subtypes. It was found that **14** at 100 nM right-shifted the concentration−response curve to the potent A_1AR agonist N^6-cyclopentyladenosine (CPA) in the cyclic AMP functional assay (inhibitory) in a parallel manner (Figure 4A). The EC_{50} values of CPA in the absence and presence of compound **14** at 100 nM were 1.5 ± 0.3 and 45 ± 17.2 nM, respectively. The activation of the A_1AR is also reported to stimulate mobilization of intracellular Ca^{2+} in various cells, which is likely dependent on $G\beta\gamma$ subunits.[30] CPA stimulated Ca^{2+} mobilization with an EC_{50} of 57.5 ± 16.8 nM (Figure 4B), and compound **14** at a concentration (10 nM) lower than its K_i value both right-shifted the CPA curve and suppressed the maximal effect, suggesting qualitative pharmacological differences between the conjugates and monomers. In contrast, the monomer XAC **3** at 10 nM or 100 nM (Figure 4C) inhibited CPA-induced calcium mobilization in A_1AR-expressing CHO cells in a competitive fashion. In the presence of 100 nM XAC, CPA displayed an EC_{50} of 346 ± 58 nM, corresponding to a 6-fold reduced potency.

A representative conjugate **14** was examined as an antagonist in a functional adenylate cyclase assay at the G_s-coupled $hA_{2B}AR$ expressed in CHO cells.[19] The multivalent xanthine at a concentration of 10 nM produced an 18-fold right-shift of the NECA response curve (Figure 5).

DISCUSSION

In this study, we used the Cu(I)-catalyzed alkyne−azide click reaction as a chemically and biologically effective means of linking a functionalized congener of a potent AR antagonist to PAMAM dendrimers. Click chemistry was previously used to link small molecular AR agonists to dendrimeric structures,[30] but this is the first systematic probing of PAMAM dendrimer conjugates of small-molecular AR antagonists. As with the agonist conjugates, high affinity of the GLiDe conjugates was achieved. The efficiency of the click reaction readily allowed a high degree of substitution in the dendrimer derivatives that could be varied by the proportion of PEG-alkyne or XAC-alkyne to dendrimer molecules that was added to the reaction.

The effectiveness of PEGylation of polymeric drug conjugtes to enhance polymer architecture, self-assembly, and bioavailability has been demonstrated.[31] In the present conjugates of a hydrophobic xanthine pharmacophore, water-solubility was increased, and the characteristic aggregation of PAMAM dendrimers was reduced by the presence of PEG groups. We compared different degrees of loading of the GPCR ligand and of PEG groups to the dendrimeric carrier. The ability to bind the ARs was not prevented by attached PEG groups (each approximately 43 oxoethylene units in length), similar to previous findings with AR agonist GLiDe conjugates.[32]

More highly XAC-substituted dendrimer derivatives prepared by click chemistry were particularly potent in binding to the ARs, with some selectivity for the $hA_{2A}AR$ subtype in comparison to that for hA_1 and hA_3ARs. There was also indication of high potency antagonist activity at the hA_{2B} for a representative derivative **14**. Nearly nanomolar affinities in binding to three AR subtypes were attained in the most highly XAC-substituted conjugates, with affinity enhancement for the conjugates versus monomer **4** being most pronounced for the A_3AR. The phenomenon of progressively increased affinity upon greater xanthine substitution was evident in both series of high and low PEG content. However, the function of the gain in AR affinity with increasing xanthine content did vary somewhat with different degrees of PEG substitution. The conjugates with an average of 22 PEG units, corresponding to a total of 44,000 D added to the molecular weight of the PAMAM, were initially weaker in binding to ARs than the 8 PEG series but gained more rapidly with increasing xanthine substitution. The molecular weights of the highly substituted conjugates **14** and **18** were ~78 and ~53 kD, respectively. Thus, the PEG content of these two conjugates amounted to approximately 56% and 30%, respectively, of the total mass.

Substitution with additional prosthetic groups of varied structure attached either by click or amide-forming reactions did not appreciably alter the binding affinity. For example, two sterically bulky NIR dyes with absorption in the range of 800 and 700 nm that are used in imaging in vivo[33] were incorporated in conjugates with retention of high receptor binding affinity. For example, conjugate **25** is identical in A_3AR affinity to its precursor and contains an average of 4 equivalents of IR dye 800CW, which is excited at 785 nm and fluorescence can be measured at 830 nm,

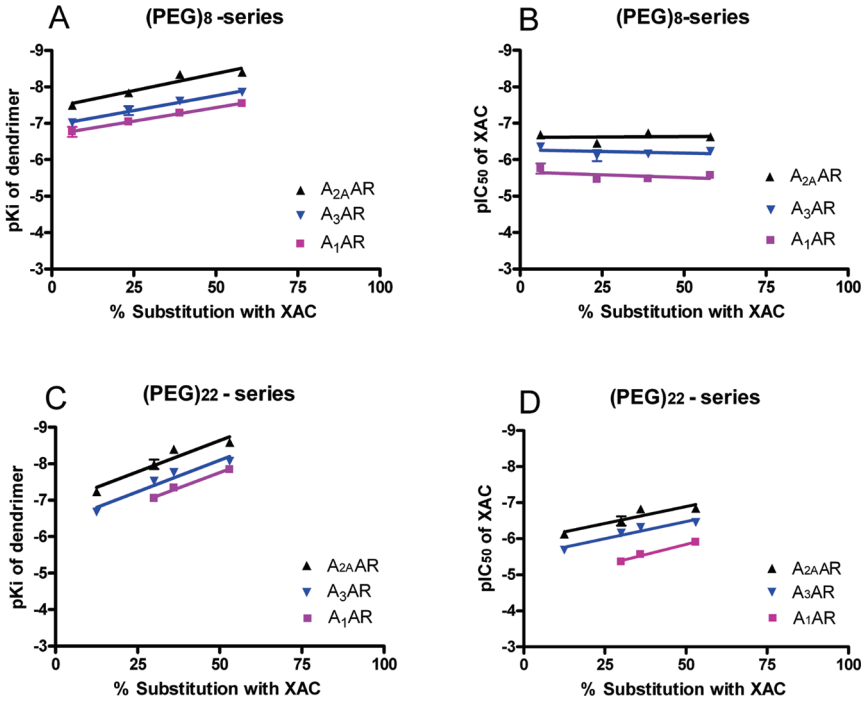

Figure 3. Inhibition of radioligand binding by dendrimer nucleoside conjugates in membranes of CHO cells expressing the hA_1 and A_3 ARs and HEK-293 cells expressing the $hA_{2A}AR$ showing dependence on the degree of substitution with the AR antagonist pharmacophore (XAC). Two methods of calculating the data were used on the basis of the definition of antagonist concentration: according to the concentration of the dendrimer (A,C) or according to the concentration of the pendant xanthine moieties (B,D). The conjugates were generally slightly selective for the $hA_{2A}AR$ in comparison to the hA_1 and hA_3ARs.

which is suitable for whole body imaging in small animals. Such GLiDe conjugates might eventually be useful for in vivo diagnostic imaging of tissue overexpressing ARs, such as occurs in disease states.[34] Thus, the ability to substitute the dendrimer with chemically diverse prosthetic groups in this series of AR conjugates allows for considerable structural breadth for probing and possibly targeting of the conjugates to the ARs or to cells having specific markers on the surface.

The advantages of nanocarriers for small molecular weight drugs include enhanced pharmacokinetic and pharmacodynamic properties. Our approach derivatizes a small molecular GPCR ligand for coupling to a carrier but allows it to remain active while covalently attached.[1,15] Internalization is not needed or desired because the binding sites of the receptors are accessible only from the extracellular medium. Molecular modeling indicated that flexible PAMAM dendrimers are able to spread over the cell surface, and purine conjugates of G3 dendrimers and higher generations can readily adopt a conformation that can bridge multiple GPCR binding sites.[8] In the theoretical model of a dendrimer conjugate of an $A_{2A}AR$ agonist bridging adjacent binding sites of an $A_{2A}AR$ homodimer, the distance between the two sites was ~35 Å, which was easily accommodated by two intermediate-spaced arms of the G3 dendrimer in a nonextended conformation. This roughly spherical G3 dendrimer conjugate had a diameter of ~67 Å, and the xanthine moieties of the present G4 conjugates in a non-extended conformation might be expected to achieve an estimated maximum separation of ~80 Å. This distance would be increased by stretching of the dendrimer over a surface.[35]

GPCRs tend not to be evenly or randomly distributed over the surface of a given cell; we demonstrated this for the hA_3AR expressed heterologously in CHO cells.[2] 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 an agonist to both protomers does not have equivalent pharmacological effects.[36] Thus, a multivalent GPCR ligand might display unanticipated, complex effects.[5] There is a lack of GPCR ligands that are designed specifically to interact with dimeric and oligomeric receptors. We do not have effective ligand tools for separating the effects of one receptor dimeric combination from another. The approach of mixing ligands for two different receptors[3,37,41] promises to be a means of achieving selectivity for receptors in a given tissue, on the basis of 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. Nevertheless, providing tools for studying these receptor aggregates is one of the objectives of this study. The multivalent binding to other types of cell surface receptors has been explored through sytematic structural modification of the carrier polymer.[42]

There is evidence here for multivalent binding of the same conjugate to more than one AR site. The fact that the AR affinity rose substantially with increased xanthine content, even when calculating IC_{50} values according to the concentration of the pharmacophore rather than the dendrimer as shown in Figure 3D, suggests that the same GLiDe conjugate molecule may bind to multiple AR binding sites. The interaction of the multivalent GLiDe conjugates can now be studied in cells expressing varying levels of a given AR or with other receptors coexpressed.

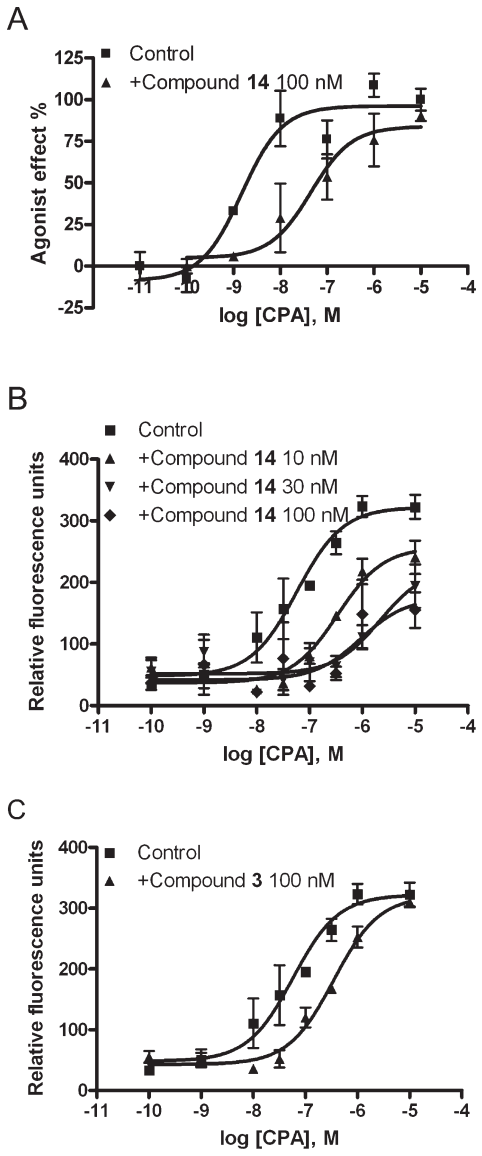

Figure 4. Antagonist activity of compound **14** in the inhibition of forskolin-stimulated cyclic AMP accumulation induced by agonist CPA (A) and the inhibition by **14** of CPA-induced calcium mobilization (B) in CHO cells stably expressing the hA_1AR. Part C shows the comparable inhibition by XAC **3** of CPA-induced calcium mobilization in hA_1AR-expressing CHO cells. Each curve is representative of three determinations. The EC_{50} values are listed in the text.

In previous studies, we have noted that the pharmacological profile of GLiDe conjugates can differ from that of the monomeric ligands both quantitatively and qualitatively.[5,11] For example, the dendrimer conjugates of AR agonists have displayed a shift in the AR subtype selectivity or in the correspondence between the binding and functional potencies.[2,11] Here, we have detected a qualitative difference in the A_1AR antagonistic response between a potent, multivalent xanthine conjugate and its corresponding monomer. Conjugate **14** acted as a competitive antagonist of the $G_{i\alpha}$-dependent effect of inhibition of adenylate cyclase, but at comparable concentrations acted as a noncompetitive inhibitor of calcium mobilization, which is likely independent of $G_{i\alpha}$. The functional effects at other AR subtypes were not determined here and will be the subject of further studies.

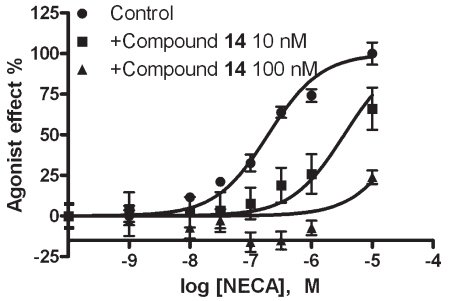

Figure 5. Antagonist activity of compound **14** in the stimulation of cyclic AMP accumulation induced by agonist NECA in CHO cells stably expressing the $hA_{2B}AR$. Each curve is representative of three determinations. The EC_{50} values for NECA in the absence and presence of 10 nM **14** are 194 nM and 3.46 μM, respectively.

In conclusion, we have synthesized and characterized pharmacologically a series of multivalent dendrimeric derivatives of a potent strategically functionalized AR antagonist. GLiDe conjugates provide different quantitative and qualitative pharmacological properties than do monomers. Prosthetic groups designed for therapeutic targeting or reporter groups for diagnostic purposes might be introduced. Various therapeutic applications[12] may be proposed for multivalent conjugates of AR antagonists. In vivo studies will be needed to fully characterize these potent ligands biologically and determine their advantage over monomeric drugs.

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR and mass spectra of representative compounds **14** and **18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AR, adenosine receptor; cAMP, adenosine 3′,5′-cyclic phosphate; CGS21680, 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyladenosine; DIPEA, diisopropylethylamine;

DMEM, Dulbecco's modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; EDC, *N*-ethyl-*N*′-dimethylaminopropylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; GLiDe, GPCR ligand−dendrimer; GPCR, G protein-coupled receptor; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I-AB-MECA, 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine; IB-MECA, N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; MS, mass spectrometry; NECA, 5′-*N*-ethylcarboxamidoadenosine; NMR, nuclear magnetic resonance; NIR, near-infrared; PAMAM, polyamidoamine; PEG, polyethyleneglycol; R-PIA, *R*-N^6-(2-phenylisopropyl)adenosine.

REFERENCES

(1) Kim, Y., Hechler, B., Klutz, A., Gachet, C., and Jacobson, K. A. (2008) Toward multivalent signaling across G protein-coupled receptors from poly(amidoamine) dendrimers. *Bioconjugate Chem. 19*, 406–411.

(2) Klutz, A. M., Gao, Z. G., Lloyd, J., Shainberg, A., and Jacobson, K. A. (2008) Enhanced A_3 adenosine receptor selectivity of multivalent nucleoside-dendrimer conjugates. *J. Nanobiotechnol. 6*, 12.

(3) Tosh, D. K., Yoo, L. S., Chinn, M., Hong, K., Kilbey, S. M., Barrett, M. O., Fricks, I. P., Harden, T. K., Gao, Z. G., and Jacobson, K. A. (2010) Polyamidoamine (PAMAM) dendrimer conjugates of "clickable" agonists of the A_3 adenosine receptor and coactivation of the $P2Y_{14}$ receptor by a tethered nucleotide. *Bioconjugate Chem. 21*, 372–384.

(4) Overington, J. (2006) How many drug targets are there?. *Nat. Rev. Drug. Discovery 5*, 993–996.

(5) Jacobson, K. A. (2010) GPCR ligand-dendrimer (GLiDe) conjugates: future smart drugs?. *Trends Pharmacol. Sci. 31*, 575–579.

(6) Ferré, S., Baler, R., Bouvier, M., Caron, M. G., Devi, L. A., Durroux, T., Fuxe, K., George, S. R., Javitch, J. A., Lohse, M. J., Mackie, K., Milligan, G., Pfleger, K. D., Pin, J. P., Volkow, N. D., Waldhoer, M., Woods, A. S., and Franco, R. (2009) Building a new conceptual framework for receptor heteromers. *Nat. Chem. Biol. 5*, 131–134.

(7) Nakata, H., Suzuki, T., Namba, K., and Oyanagi, K. (2010) Dimerization of G protein-coupled purinergic receptors: increasing the diversity of purinergic receptor signal responses and receptor functions. *J. Recept. Signal Transduction 30*, 337–346.

(8) Ivanov, A. A., and Jacobson, K. A. (2008) Molecular modeling of a PAMAM-CGS21680 dendrimer bound to an A_{2A} adenosine receptor homodimer. *Bioorg. Med. Chem. Lett. 18*, 4312–4315.

(9) de Castro, S., Maruoka, H., Hong, K., Kilbey, S. M., Costanzi, S., Hechler, B., Gachet, C., Harden, T. K., and Jacobson, K. A. (2010) Functionalized congeners of $P2Y_1$ receptor antagonists: 2-Alkynyl (N)-methanocarba 2′-deoxyadenosine 3′,5′-bisphosphate analogues and conjugation to a polyamidoamine (PAMAM) dendrimer carrier. *Bioconjugate Chem. 21*, 1190–1205.

(10) Maeda, H., Bharate, G. Y., and Daruwalla, J. (2009) Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. *Eur. J. Pharm. Biopharm. 71*, 409–419.

(11) Keene, A. M., Balasubramanian, R., Lloyd, J., Shainberg, A., and Jacobson, K. A. (2010) Multivalent dendrimeric and monomeric adenosine agonists attenuate cell death in HL-1 mouse cardiomyocytes expressing the A_3 adenosine receptor. *Biochem. Pharmacol. 80*, 188–196.

(12) Jacobson, K. A., and Gao, Z. G. (2006) Adenosine receptors as therapeutic targets. *Nature Rev. Drug Discovery 5*, 247–264.

(13) Müller, C., and Jacobson, K. A. (2011) Xanthines as Adenosine Receptor Antagonists. In Methylxanthines. *In Handbook of Experimental Pharmacology* (Fredholm, B.B., Ed.) Vol. 200, pp 151−199, Springer, New York.

(14) Baraldi, P. G., Tabrizi, M. A., Gessi, S., and Borea, P. A. (2008) Adenosine receptor antagonists: translating medicinal chemistry and pharmacology into clinical utility. *Chem. Rev. 108*, 238–263.

(15) Jacobson, K. A., Kirk, K. L., Padgett, W. L., and Daly, J. W. (1985) Functionalized congeners of 1,3-dialkylxanthines: preparation of analogues with high affinity for adenosine receptors. *J. Med. Chem. 28*, 1334–1340.

(16) Jacobson, K. A. (2009) Functionalized congener approach to the design of ligands for G protein−coupled receptors (GPCRs). *Bioconjugate Chem. 20*, 1816–1835.

(17) van Dijk, M., Rijkers, D. T. S., Liskamp, R. M. J., van Nostrum, C. F., and Hennink, W. E. (2009) Synthesis and applications of biomedical and pharmaceutical polymers via click chemistry methodologies. *Bioconjugate Chem. 20*, 2001–2016.

(18) Goddard-Borger, E. D., and Stick, R. V. (2007) An efficient, inexpensive, and shelf-stable diazotransfer reagent: Imidazole-1-sulfonyl azide hydrochloride. *Org. Lett. 9*, 3797–3800.

(19) Adachi, H., Palaniappan, K. K., Ivanov, A. A., Bergman, N., Gao, Z. G., and Jacobson, K. A. (2007) Structure-activity relationships of 2, N^6,5′-substituted adenosine derivatives with potent activity at the A_{2B} adenosine receptor. *J. Med. Chem. 50*, 1810–1827.

(20) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248–254.

(21) Klotz, K. N., Lohse, M. J., Schwabe, U., Cristalli, G., Vittori, S., and Grifantini, M. (1989) 2-Chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA)-a high affinity agonist radioligand for A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol. 340*, 679–683.

(22) Jarvis, M. F., Schutz, R., Hutchison, A. J., Do, E., Sills, M. A., and Williams, M. (1989) [^{3}H]CGS 21680, an A_2 selective adenosine receptor agonist directly labels A_2 receptors in rat brain tissue. *J. Pharmacol. Exp. Ther. 251*, 888–893.

(23) Olah, M. E., Gallo-Rodriguez, C., Jacobson, K. A., and Stiles, G. L. (1994) ^{125}I-4-Aminobenzyl-5′-N-methylcarboxamidoadenosine, a high affinity radioligand for the rat A_3 adenosine receptor. *Mol. Pharmacol. 45*, 978–982.

(24) Middleton, R. J., and Kellam, B. (2005) Fluorophore-tagged GPCR ligands. *Curr. Opin. Chem. Biol. 9*, 517–525.

(25) Kumar, T. S., Mishra, S., Deflorian, F., Yoo, L. S., Phan, K., Kecskés, M., Szabo, A., Shinkre, B. A., Gao, Z. G., Trenkle, W. C., and Jacobson, K. A. (2011) Molecular probes for the A_{2A} adenosine receptor based on a pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-c]pyrimidine scaffold. *Bioorg. Med. Chem. Lett. 21*, 2740–2745.

(26) Baraldi, P. G., Cacciari, B., Moro, S., Romagnoli, R., Ji, X.-d., Jacobson, K. A., Gessi, S., Borea, P. A., and Spalluto, G. (2001) Fluorosulfonyl- and bis-(b-chloroethyl)amino-phenylamino functionalized pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine derivatives: Irreversible antagonists at the human A_3 adenosine receptor and molecular modeling studies. *J. Med. Chem. 44*, 2735–2742.

(27) Zwier, J. M., Roux, T., Cottet, M., Durroux, T., Douzon, S., Bdioui, S., Gregor, N., Bourrier, E., Oueslati, N., Nicolas, L., Tinel, N., Boisseau, C., Yverneau, P., Charrier-Savournin, F., Fink, M., and Trinquet, E. (2010) A fluorescent ligand-binding alternative using Tag-lite® technology. *J. Biomol. Screen. 15*, 1248–1260.

(28) Englert, M., Quitterer, U., and Klotz, K. N. (2002) Effector coupling of stably transfected human A_3 adenosine receptors in CHO cells. *Biochem. Pharmacol. 64*, 61–65.

(29) Jacobson, K. A., Park, K. S., Jiang, J. -l., Kim, Y. C., Olah, M. E., Stiles, G. L., and Ji, X. d. (1997) Pharmacological characterization of novel A_3 adenosine receptor-selective antagonists. *Neuropharmacology 36*, 1157–1165.

(30) Ethier, M. F., and Madison, J. M. (2006) Adenosine A_1 receptors mediate mobilization of calcium in human bronchial smooth muscle cells. *Am. J. Respir. Cell Mol. Biol. 35*, 496–502.

(31) Tosh, D. K., Chinn, M., Yoo, L. S., Kang, D. W., Luecke, H., Gao, Z. G., and Jacobson, K. A. (2010) 2-Dialkynyl derivatives of (N)-methanocarba nucleosides: 'Clickable' A_3 adenosine receptor-selective agonists. *Bioorg. Med. Chem. 18*, 508–517.

(32) Joralemon, M. J., McRae, S., and Emrick, T. (2010) PEGylated polymers for medicine: from conjugation to self-assembled systems. *Chem. Commun. (Cambridge, U.K.) 46*, 1377–1393.

(33) Kim, Y., Hechler, B., Gao, Z. G., Gachet, C., and Jacobson, K. A. (2009) PEGylated dendritic unimolecular micelles as versatile carriers for ligands of G protein-coupled receptors. *Bioconjugate Chem. 20*, 1888–1898.

(34) Adams, K. E., Ke, S., Kwon, S., Liang, F., Fan, Z., Lu, Y., Hirschi, K., Mawad, M. E., Barry, M. A., and Sevick-Muraca, E. M. (2007) Comparison of visible and near-infrared wavelength excitable fluorescent dyes for molecular imaging of cancer. *J. Biomed. Opt. 12*, 024017.

(35) Ochaion, A., Bar-Yehuda, S., Cohen, S., Barer, F., Patoka, R., Amital, H., Reitblat, T., Reitblat, A., Ophir, J., Konfino, I., Chowers, Y., Ben-Horin, S., and Fishman, P. (2009) The anti-inflammatory target A_3 adenosine receptor is overexpressed in rheumatoid arthritis, psoriasis and Crohn's disease. *Cell. Immunol. 258*, 115–122.

(36) Tomalia, D. A., Naylor, A. M, and Goddard, W. A. (1990) Starburst dendrimers: molecular level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter. *Angew. Chem., Int. Ed. 29*, 138–175.

(37) Han, Y., Moreira, I. S., Urizar, E., Weinstein, H., and Javitch, J. A. (2009) Allosteric communication between protomers of dopamine class A GPCR dimers modulates activation. *Nature Chem. Biol. 5*, 688–695.

(38) Portoghese, P. S., Ronsisvalle, G., Larson, D. L., Yim, C. B., Sayre, L. M., and Takemori, A. E. (1982) Opioid agonist and antagonist bivalent ligands as receptor probes. *Life Sci. 31*, 1283–1286.

(39) Morphy, R., and Rankovic, Z. (2005) Designed multiple ligands. An emerging drug discovery paradigm. *J. Med. Chem. 48*, 6523–6565.

(40) Soriano, A., Ventura, R., Molero, A., Hoen, R., Casadó, V., Cortés, A., Fanelli, F., Albericio, F., Lluís, C., Franco, R., and Royo, M. (2009) Adenosine A_{2A} receptor-antagonist/dopamine D_2 receptor-agonist bivalent ligands as pharmacological tools to detect A_{2A}-D2 receptor heteromers. *J. Med. Chem. 52*, 5590–5602.

(41) Jacobson, K. A., Xie, R., Young, L., Chang, L., and Liang, B. T. (2000) A novel pharmacological approach to treating cardiac ischemia: binary conjugates of A_1 and A_3 adenosine receptor agonists. *J. Biol. Chem. 275*, 30272–30279.

(42) Berque-Bestel, I., Lezoualc'h, F., and Jockers, R. (2008) Bivalent ligands as specific pharmacological tools for G protein-coupled receptor dimers. *Curr. Drug Discovery Technol. 5*, 312–318.

(43) Cairo, C. W., Gestwicki, J. E., Kanai, M., and Kiessling, L. L. (2002) Control of multivalent interactions by binding epitope density. *J. Am. Chem. Soc. 124*, 1615–1619.