Modulation of G protein-coupled adenosine receptors by strategically functionalized agonists and antagonists immobilized on gold nanoparticles

P. Suresh Jayasekara · Khai Phan · Dilip K. Tosh ·
T. Santhosh Kumar · Steven M. Moss · Guofeng Zhang ·
Joseph J. Barchi Jr. · Zhan-Guo Gao ·
Kenneth A. Jacobson

Received: 27 July 2012 / Accepted: 30 October 2012 / Published online: 20 November 2012
© Springer Science+Business Media Dordrecht (outside the USA) 2012

Abstract Gold nanoparticles (AuNPs) allow the tuning of pharmacokinetic and pharmacodynamic properties by active or passive targeting of drugs for cancer and other diseases. We have functionalized gold nanoparticles by tethering specific ligands, agonists and antagonists, of adenosine receptors (ARs) to the gold surface as models for cell surface interactions with G protein-coupled receptors (GPCRs). The AuNP conjugates with chain-extended AR ligands alone (PEGylated nucleosides and nonnucleosides, anchored to the Au via thioctic acid) were found to be insoluble in water due to hydrophobic entities in the ligand. Therefore, we added a second, biologically inactive pendant moiety to increase the water solubility, consisting of a PEGylated chain terminating in a carboxylic or phosphate group. The purity and stability of the immobilized biologically active

ligand were examined by ultrafiltration and HPLC. Pharmacological receptor binding studies on these GPCR ligand-derivatized AuNPs (2–5 nm in diameter), performed using membranes of mammalian cells stably expressing human A₁, A_{2A}, and A₃ARs, showed that the desired selectivity was retained with *K_i* values (nanomolar) of A₃AR agonist **21b** and A_{2A}AR antagonists **24** and **26a** of 14 (A₃), 34 (A_{2A}), and 69 (A_{2A}), respectively. The corresponding monomers displayed *K_i* values of 37, 61, and 1,420 nM, respectively. In conclusion, we have synthesized stable, water-soluble AuNP derivatives of tethered A₃ and A_{2A}AR ligands that retain the biological properties of their monomeric ligands and are intended for therapeutic and imaging applications. This is the first prototypical application to gold carriers of small molecule (nonpeptide) GPCR ligands, which are under investigation for treatment of cancer and inflammatory diseases.

Electronic supplementary material The online version of this article (doi:10.1007/s11302-012-9338-z) contains supplementary material, which is available to authorized users.

P. S. Jayasekara · K. Phan · D. K. Tosh · T. S. Kumar ·
S. M. Moss · Z.-G. Gao · K. A. Jacobson (✉)
Molecular Recognition Section, Laboratory of Bioorganic
Chemistry, National Institute of Diabetes and Digestive and
Kidney Diseases, National Institutes of Health,
Bldg. 8A, Rm. B1A-19,
Bethesda, MD 20892-0810, USA
e-mail: kajacobs@helix.nih.gov

G. Zhang
Biomedical Engineering and Physical Science Shared Resource,
NIBIB, National Institutes of Health,
Bethesda, MD 20892, USA

J. J. Barchi Jr.
Chemical Biology Laboratory, Center for Cancer Research,
Frederick National Laboratory for Cancer Research,
Frederick, MD 21702-1201, USA

Keywords G protein-coupled receptor · Nanoparticle ·
Nucleoside · Adenosine · Radioligand binding

Abbreviations

AR	Adenosine receptor
DLS	Dynamic light scattering
DMSO	Dimethylsulfoxide
EDC	<i>N</i> -ethyl- <i>N</i> '-dimethylamino-propylcarbodiimide
cAMP	Adenosine 3',5'-cyclic phosphate
CHO	Chinese hamster ovary
Cl-IB-MECA	2-Chloro- <i>N</i> ⁶ -(3-iodobenzyl)-5'- <i>N</i> -methylcarboxamido-adenosine
DCC	<i>N,N</i> '-dicyclohexylcarbodiimide
DMAP	4-Dimethylaminopyridine
DCM	Dichloromethane

DIEA	Diisopropylethylamine
DMF	Dimethylformamide
DMEM	Dulbecco's modified Eagle's medium
AuNP	Gold nanoparticle
GPCR	G protein-coupled receptor
HEK	Human embryonic kidney
CGS21680	2-[<i>p</i> -(2-carboxyethyl)phenyl-ethylamino]-5'- <i>N</i> -ethylcarboxamido-adenosine
MS	Mass spectrometry
NECA	5'- <i>N</i> -ethylcarboxamidoadenosine
PTP	Pyrazolo[4,3- <i>e</i>][1, 2, 4]triazolo [1,5- <i>c</i>]pyrimidine-5-amine
PyBOP	Benzotriazol-1-yloxy-tripyrrolidino-phosphonium hexafluorophosphate
SPB	Surface plasmon absorption band
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
I-AB-MECA	<i>N</i> ⁶ -(4-amino-3-iodobenzyl)-adenosine-5'- <i>N</i> -methyluronamide
PEG	Polyethylene glycol
PIA	<i>N</i> ⁶ -(2-phenylisopropyl)adenosine
Tris	2-amino-2-hydroxymethyl-propane-1, 3-diol

Introduction

Gold nanoparticles (AuNPs) are being developed as cancer therapeutic and diagnostic agents [1]. The ability to control the size and shape of these particles allows the tuning of their pharmacokinetic and pharmacodynamic properties in passive targeting, and the option of tethering specific ligands for cell surface receptors to the functionalized gold surface facilitates active targeting [2–4]. The advantages of the application of AuNPs for therapeutics and diagnostics are numerous and potentially applicable to many diseases. First, AuNPs can circulate systemically in the blood stream due to their nanometer size, and AuNPs are considered excellent candidates as drug delivery systems due to their biocompatibility. Secondly, they can easily be coated with multiple copies of biological ligands such as DNA, small interfering RNA, and drugs through several mechanisms (e.g., bioconjugation, noncovalent interactions, and direct surface bonding) [5]. In addition, immobilization of small molecules on a gold surface can be applied to imaging, plasmon resonance-based labeling, and optical and electrochemical sensing in cancer therapy and several other disease states (viz., Alzheimer's disease, AIDS, hepatitis, tuberculosis, arthritis, and diabetes) [6, 7]. The cargo of the AuNPs may also be cytotoxic agents, for example in cancer chemotherapy, or other moieties for altering the fate of the particle in vivo or altering its ability to cross cell membranes [8]. A particularly clever application of AuNP conjugates is in thermoablation of tumor tissue by Au nanoshells,

which are concentrated in the diseased tissue followed by irradiation to elevate the temperature [9]. AuNPs were used to interrogate the mechanistic pathways involved in killing cancer cells [10]. Also, because the shape of the particle determines optical properties, it is possible to apply such particles to in vivo imaging, for example, based on absorption in the near infrared (NIR) range [9]. Design parameters are being explored to obtain biocompatible AuNPs to serve as carriers of small molecules or biomacromolecules.

G protein-coupled receptors (GPCRs), which modulate intracellular pathways to alter cell survival, proliferation, and other functions, have recently become a focus in relation to the treatment of cancer [11]. GPCRs are involved in a wide range of diseases and serve as the basis of at least one third of the pharmaceuticals on the market. There are four known subtypes of adenosine receptors (ARs), which belong to the superfamily of GPCRs, referred to as A₁, A_{2A}, A_{2B}, and A₃—each of which has a unique pharmacological profile, tissue distribution, and combination of effects [12, 13]. An agonist of the A₃AR is currently in clinical trials for hepatocellular carcinoma [13, 14]. Fishman et al. reported that growth of melanoma cells can be inhibited by activation of A₃AR via deregulation of the NFκB and Wnt pathways [14]. They demonstrated that the small molecule nucleoside agonists IB-MECA **1** and Cl-IB-MECA **2** (Fig. 1), both invented in our laboratory, inhibit the growth of B16-F10 melanoma cells via activation of A₃AR both in vitro and in vivo, and among these, **2** has already entered phase II clinical trials for hepatocellular carcinoma [14, 43]. Additionally, A_{2A}AR agonists have anti-inflammatory properties and are used in cardiac imaging [13, 25]. A_{2A}AR antagonists are potentially useful in the treatment of Parkinson's disease, progressive neurodegeneration, and cancer [13]; cerebroprotection is observed either as a result of A_{2A}AR antagonism or gene deletion [13, 15].

A recurrent problem in the development of agonist and antagonist ligands for GPCRs is the widespread occurrence of a given receptor leading to side effects at undesired sites of action. Thus, there is a need for enhancing the selectivity of GPCR ligands for specific tissues, organs, or signaling pathways [16]. Devices and drugs that can be delivered with high accuracy to maximize efficacy and minimize side effects are needed. Thus, new methods for designing GPCR modulators that function as “smart drugs” are required. One possibility could be GPCR ligand-functionalized AuNPs, where proper adjustment of their pharmacokinetic properties may reduce off-target effects.

In this study, we explored the feasibility of using functionalized AuNPs as carriers for small GPCR ligands. Unlike the abovementioned applications of AuNPs in cancer therapy, their use in targeting cell surface GPCRs would not require dissociation of the therapeutic moiety from the carrier. In fact, the linkage of the GPCR ligand could be

Fig. 1 High affinity and chemically functionalized ligands for various ARs of interest. Agonists IB-MECA (1), Cl-IB-MECA (2), MRS 3558 (3) and 4 of the A₃AR, selective agonist 5 and antagonist 6 of the A_{2A}AR, and non-selective AR antagonist 7

designed such that activity is retained in the intact tethered particle, which would bind to the surface of cells expressing the receptor for that ligand. Internalization of the particle would be a separate process that may or may not add to the efficacy of the gold nanoconjugate.

Materials and methods

Chemical synthesis

All reagents and solvents (regular and anhydrous) were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA), unless noted, and used without further purification. Reactions were conducted under an atmosphere of nitrogen whenever anhydrous solvents were used. All reactions to prepare small molecules were monitored by thin layer chromatography using silica gel-coated plates with a fluorescence indicator which were visualized: (a) under UV light, (b) by dipping in a mixture of anisaldehyde (2.5 mL)/conc. H₂SO₄ (5 mL)/methanol (425 mL), or (c) by dipping the plate in a solution of ninhydrin (0.3 g in 100 mL EtOH, containing AcOH, 1.3 mL) followed by heating. Silica gel column chromatography was performed with silica gel (SiO₂, 200–400 mesh, 60 Å, Sorbent Technologies, Atlanta, GA, USA) using moderate air pressure. Evaporation of solvents was carried out under reduced pressure at a temperature below 50 °C. After column chromatography, appropriate fractions were pooled, evaporated, and dried at high vacuum for at least 12 h to give the desired products in high purity. ESI-high resolution mass spectroscopic measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with

polyalanine. Observed mass accuracies are those expected on the basis of known performance of the instrument as well as the trends in masses of standard compounds observed at intervals during the series of measurements. Reported masses are observed masses uncorrected for this time-dependent drift in mass accuracy.

The synthetic intermediates and AR ligands (including AuNP conjugates) were characterized by ¹H NMR. The spectra were recorded on a Bruker 400 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane or using deuterated solvent as the internal standard (δ H: CDCl₃ 7.26 ppm). All *J* values are reported in Hertz (Hz). Electronic absorption spectra were recorded using an HP 8453 diode array spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The spectra were collected using quartz cuvettes (Spectrocell, Inc., Oreland, PA, USA) with 0.5 cm optical path length. The prepared AuNPs were characterized structurally by examination under a FEI Tecnai12 transmission electron microscope operating at a beam energy of 120 keV. Images were acquired by using a 2 k×2 k cooled CCD camera from Gatan (Warrendale, PA, USA). Samples for transmission electron microscopy (TEM) were prepared by spreading a drop of the AuNP dispersion onto an ultrathin carbon support film on a fine mesh Cu grid (400 mesh) and letting it dry. The nanoparticles were imaged with a 1.8-nm pixel size. Dynamic light scattering measurements were carried out in a DynaPro NanoStar (Wyatt Technology, Santa Barbara, CA, USA). We purchased dialysis membranes (Spectra/Pore Membrane, MW cutoff 3,500, flat width 18 mm) from Spectrum Labs (Rancho Dominguez, CA, USA). The Amicon Ultra-15 centrifugal filter unit (30 K MW cutoff) was purchased from Millipore Ltd. (Billerica, MA, USA).

Synthesis of monomeric AR ligands, linkers, and solubilizing moieties

Compound 12

(R)-Thioctic acid (0.19 g, 0.95 mmol, TA, also known as α -lipoic acid), N_3 -(PEG) $_{10}$ -NH $_2$ (Aldrich, 0.50 g, 0.95 mmol), 4-(*N,N*-dimethylamino)-pyridine (0.03 g, 0.28 mmol), and CH $_2$ Cl $_2$ (20 mL) were added in a 100-mL round bottom flask, which was cooled to 0 °C. *N,N'*-dicyclohexylcarbodiimide (DCC, 0.19 g, 0.95 mmol) was slowly added under N $_2$ with stirring. The above mixture was stirred at 0 °C for 2 h, then warmed to room temperature, and further stirred overnight. The reaction mixture was filtered through Celite and the filter rinsed with ethyl acetate. The filtrate was evaporated, and the residue was chromatographed on silica gel with CHCl $_3$ and then 10:1 CH $_2$ Cl $_2$ /MeOH as eluent to give 0.66 mmol (0.47 mg) of homogeneous TA-(PEG) $_{10}$ -N $_3$ **12** (yield 70 %). ^1H NMR (400 MHz, CDCl $_3$): δ 6.17 (br s, 1H), 3.55–3.65 (m, PEG H), 3.51 (m, 3H), 3.40 (m, 2H), 3.34 (m, 2H), 3.05–3.15 (m, 2H), 2.42 (m, 1H), 2.14 (t, 2H, $J=7.6$), 1.87 (m, 1H), 1.62 (m, 4H), 1.42 (m, 2H). m/z (M+ESI MS) found: 715.3640; calc for C $_{30}$ H $_{58}$ N $_4$ O $_{11}$ S $_2$: 715.3622.

Compound 13

To a mixture of TA-PEG-N $_3$ (**12**) (11.4 mg, 0.016 mmol) and alkyne **4** (6 mg, 0.011 mmol, A $_3$ AR agonist) [17] in a 0.50 mL of (1:1) mixture of $^t\text{BuOH}$ and water was added tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA, 1 mg, 0.001 mmol) and freshly prepared 1 M aqueous sodium ascorbate solution (11.7 μL , 0.011 mmol) followed by 7.5 % aqueous copper sulfate pentahydrate solution (19.4 μL , 0.005 mmol). The reaction mixture was stirred overnight at room temperature, the solvent was evaporated, and the residue was purified on a flash silica gel chromatography column (CH $_2$ Cl $_2$ /MeOH=30:1) to give A $_3$ AR agonist monomer **13** for Au complexation (7.5 mg, 55 %). ^1H NMR (400 MHz, MeOD): δ 8.08 (s, 1H), 7.83 (s, 1H), 7.41 (s, 1H), 7.25–7.31 (m, 2H), 5.01 (d, 1H, $J=6.7$ Hz), 4.52 (dd, 2H, $J_1=5.08$ Hz, $J_2=4.76$ Hz), 3.98 (d, 1H, $J=5.9$ Hz), 3.85 (t, 2H, $J_1=4.92$ Hz), 3.5–3.75 (m, PEG H), 3.3–3.45 (m, 2H), 3.05–3.15 (m, 2H), 2.85 (s, 3H), 2.78 (t, 2H, $J_1=7.44$ Hz), 2.51 (dd, 2H, $J_1=7.08$ Hz, $J_2=6.7$ Hz), 2.43 (m, 1H), 2.20 (dd, 2H, $J_1=7.24$ Hz, $J_2=7.36$ Hz), 2.06 (m, 1H), 1.86 (m, 4H), 1.6–1.8 (m, 8H), 1.3–1.5 (m, 2H). ^{13}C NMR (400 MHz, MeOD): δ 174.6, 173.1, 133.8, 129.7, 126.6, 125.7, 87.7, 86.0, 86.8, 76.0, 71.9, 70.2, 69.8, 69.7, 69.0, 56.2, 50.3, 50.0, 39.9, 38.7, 35.4, 34.3, 28.4, 28.3, 27.3, 27.1, 25.3, 18.2, 13.4. m/z (M + ESI MS) found: 1,247.5565; calc for C $_{53}$ H $_{88}$ N $_{12}$ O $_{16}$ S $_2$ Cl: 1,247.5571.

Compound 15

The synthesis followed a published procedure [18]. TA-(PEG) $_{10}$ -N $_3$ (**12**) (0.409 g, 0.572 mmol) and triphenylphosphine (0.211 g, 2 equiv. 0.817 mmol) were stirred with 6.3 mL of tetrahydrofuran (THF) at room temperature under a N $_2$ atmosphere for 2 h. Water (0.318 mL, 18 mmol) was added to the mixture, and stirring of the reaction mixture under N $_2$ was continued for 72 h. The solvent was evaporated, and the crude product was purified by silica gel column chromatography using first 10:1 dichloromethane (DCM)/MeOH and then 100:20:1 DCM/MeOH/Et $_3$ N as eluent. Compound **15** (0.343 g, 60 %, TA-(PEG) $_{10}$ -NH $_2$) was obtained as a yellowish liquid. ^1H NMR (300 MHz, CDCl $_3$): δ 6.48 (brs, 1H), 3.61 (m, PEG H), 3.51 (m, 2H), 3.40 (m, 1H), 3.11 (m, 2H), 2.94 (t, 2H, $J=5.2$), 2.76 (brs, 2H), 2.41 (m, 1H), 2.16 (t, 2H, $J=7.6$), 1.85 (m, 1H), 1.63 (m, 4H), 1.42 (m, 2H). m/z (M + ESI MS) found: 689.9310; calc: 689.9318.

Compound 16

TA-(PEG) $_{10}$ -NH $_2$ (**12**) (97.0 mg, 0.141 mmol), succinic anhydride (240 mg, 0.239 mmol), and dry pyridine 1.55 mL were added to round bottom flask under N $_2$. The mixture was stirred at room temperature for 17 h. The solvent was evaporated and the crude product was purified by column chromatography as the eluent (10:1 CHCl $_3$ /MeOH) to give yellowish homogeneous compound **16** (57 mg, 52 %, TA-(PEG) $_{10}$ -NH-succinoyl, a carboxylate-solubilizing moiety for Au complexation). ^1H NMR (400 MHz, CDCl $_3$): δ 7.12 (brs, 1H), 6.45 (brs, 1H), 3.61 (m, PEG H), 3.52 (m, 7H), 3.40 (m, 5H), 3.11 (m, 2H), 2.63 (dd, 2H, $J_1=6.2$, $J_2=6.8$), 2.45 (dd, 2H, $J_1=6.4$, $J_2=5.76$), 2.43 (brs, 2H), 2.17 (t, 3H, $J=7.2$), 1.88 (m, 3H), 1.63 (m, 8H), 1.42 (m, 4H). m/z (M + ESI MS) found: 787.3654; calc for C $_{34}$ H $_{63}$ N $_2$ O $_{14}$ S $_2$: 787.3721.

Compound 14

The *N*-heptynoyl derivative of XAC (**7**, xanthine amine congener, 8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine) was synthesized according to a published procedure [19]. The alkyne derivative (**7**) (3.65 mg, 5.10 μmol) and TA-PEG-N $_3$ (**12**) (2 mg, 3.01 μmol) were dissolved in 0.30 mL of a (3:1) mixture of DMF and water in a round bottom flask, and TBTA (1.01 mg, 1.02 μmol) was added. Freshly prepared sodium ascorbate (1 M, 3.72 μL , 3.70 μmol) followed by 7.5 % aqueous copper sulfate pentahydrate solution (6.16 μL , 1.86 μmol) was added to the above mixture. The reaction mixture was stirred overnight at room temperature, the solvent was evaporated, and the residue was purified on a flash silica gel chromatography column (CHCl $_3$ /MeOH=6:1) to give xanthine AR antagonist

monomer **14** for Au complexation (3.02 mg, 2.40 μmol). ^1H NMR (400 MHz, MeOD): δ 7.98 (d, 2H, $J=8.8$ Hz), 7.72 (s, 1H), 7.08 (d, 2H, $J=8.8$), 4.55 (s, 2H), 4.47 (t, 3H, $J=5.0$), 4.09 (t, 3H, $J=7.2$ Hz), 3.90 (t, 3H, $J=7.5$), 3.86 (t, 3H, $J=5.1$ Hz), 3.7–3.5 (m), 3.3–3.0 (m), 2.62 (m, 2H), 2.43 (m, 1H), 2.18 (m, 4H), 1.86–1.79 (m, 3H), 1.67–1.57 (m, 12H), 1.42 (m, 2H), 1.27 (m, 3H), 0.90 (dt, 6H, $J_1=14.4$ Hz, $J_2=7.4$ Hz). m/z (M + ESI MS) found: 1,273.6241; calc for $\text{C}_{63}\text{H}_{94}\text{N}_8\text{O}_{14}\text{S}_2\text{Na}$: 1,273.6229.

Compound 17

The PEGylated amine **15** (34 mg, 50 μmol) and **5** (CGS21680) (12.1 mg, 20.1 μmol) as a hydrochloride salt were dissolved in 1 mL DMF. Then, diisopropylethylamine (DIEA, 9 μL , 50.3 μmol) was added to the reaction mixture and stirring continued for 10 min. Then, benzotriazol-1-yloxy-tripyrrolidino-phosphonium hexafluorophosphate (PyBOP, 11.2 mg, 20.6 μmol) was added to the solution, and the mixture was stirred for 18 h at room temperature. The solvent was removed under vacuum, and the crude product was dissolved in a minimum volume (200 μL) of methanol. An excess volume (5–7 mL) of dry diethyl ether was added, and the mixture was left overnight at 4 $^\circ\text{C}$, leading to the precipitation of the product. The ether supernatant was then removed using a Pasteur pipette, and the remaining solid was dried under vacuum to obtain the pure product **17**, AR agonist monomer for Au complexation (6.01 mg, 5.0 μmol), in 25 % yield as a gummy, yellowish solid. ^1H NMR (300 MHz, DMSO): δ 8.07 (br s, 2H), 7.85, 7.78 (d (each), $J=6.8$ Hz, 4H), 5.95 (d, $J=6.4$ Hz, 1H), 4.89 (m, 1H), 4.45 (s, 1H), 4.41 (s, 1H), 3.68 (m, peg H), 3.51 (m, 2H), 3.45 (m, 1H), 3.32 (m, 6H), 2.93 (m, 6H), 2.51 (m, 2H), 2.24 (t, $J=5.4$ Hz, 2H), 2.15 (m, 2H), 1.91 (m, 1H), 1.65 (m, 4H), 1.43 (m, 2H), 0.98 (t, $J=6.8$, 3H). ^{13}C NMR (400 MHz, MeOD): δ 170.6, 156.0, 137.7, 137.5, 128.6, 128.1, 113.3, 88.5, 84.1, 73.3, 69.8, 69.2, 56.1, 45.4, 42.9, 39.9, 39.1, 38.9, 37.9, 37.5, 34.3, 33.7, 31.1, 28.4, 25.3, 23.8, 13.3. m/z (M + ESI MS) found: 1,170.5773; calc for $\text{C}_{53}\text{H}_{88}\text{N}_9\text{O}_{16}\text{S}_2$: 1,170.5790.

Compound 18

The PEGylated acid derivative **16** (12.4 mg, 15.1 μmol) and compound **6** (5.01 mg, 10.0 μmol) were dissolved in 0.9 mL DMF. DIEA (8.3 μL , 26.0 μmol) was added to the reaction mixture, and it was stirred for 10 min. Then, PyBOP (5.46 mg, 10.2 μmol) was added to the solution, and the mixture was stirred for 18 h at room temperature. The solvent was removed under vacuum, and the crude product was purified using a silica column (solvent gradient MeOH/ CH_2Cl_2 0:100 to 20:80 in 45 min). The product **18**, A_{2A}AR

antagonist monomer for Au complexation, was isolated as a white solid (9.0 mg, 7.0 μmol) in 70 % yield. ^1H NMR (400 MHz, MeOD): δ 8.0 (s, 1H), 7.83 (s, 1H), 7.41 (d, 1H, $J=3.28$ Hz), 7.0 (d, 2H, $J=8.44$ Hz), 6.81 (d, 2H, $J=8.28$ Hz), 6.63 (m, 1H), 4.38 (s, 2H), 4.33 (t, 2H, $J=6.7$ Hz), 3.65–3.52 (PEG H), 2.57 (t, 3H, $J=7.42$ Hz), 2.41 (m, 8H), 2.18 (m, 5H), 1.83 (m, 2H), 1.61 (m, 5H), 1.40 (m, 3H). m/z (M-ES MS) found: 1,244.5645; calc for $\text{C}_{57}\text{H}_{86}\text{N}_{11}\text{O}_6\text{S}_2$: 1,244.5695.

Compound 20

3-Butyne 1-monophosphate **19** (20 mg, 0.059 mmol) [20] and PEGylated azide **12** (55 mg, 78 μmol) were dissolved in a 3:1 mixture of DMF and water (3.9 mL) in a round bottom flask, and TBTA (2.0 mg, 2.0 μmol) was added. Freshly prepared aqueous sodium ascorbate (1 M, 59 μL , 59 μmol) and 7.5 % aqueous copper sulfate pentahydrate solution (150 μL , 44 μmol) were added sequentially to the above mixture. The reaction mixture was stirred overnight at room temperature, and the solvent was evaporated. The residue was purified by semipreparative HPLC as described above to obtain homogeneous compound **20**, phosphate-solubilizing monomer for Au complexation, using as mobile phase a gradient of triethylammonium acetate/ CH_3CN 100:0 to 80:20 in 20 min. ^1H NMR (400 MHz, D_2O): δ 8.01 (s, 1H), 4.62 (t, 1H), 4.10 (m, 2H), 3.99 (t, 2H, $J=5.02$ Hz), 3.71–3.62 (PEG H), 3.40 (t, 3H, $J=5.2$ Hz), 3.25 (m, 1H), 3.0 (m, 2H), 2.51 (m, 1H), 2.28 (t, 2H, $J=7.2$ Hz), 2.00 (m, 1H), 1.76 (m, 1H), 2.62 (m, 4H), 1.43 (m, 3H). m/z (M-ES MS) found: 863.3543; calc for $\text{C}_{34}\text{H}_{64}\text{N}_4\text{O}_{15}\text{S}_2\text{P}$: 863.3547.

Synthesis and characterization of AuNP conjugates

AuNP conjugate 21a

Synthesis When the same procedure as for **21b** (except redissolving the crude product in a water/methanol mixture (75:25)) was carried out using a 20:1 ratio of hydrogen tetrachloroaurate(III) tetrahydrate to each monomer **13** and **16**, a larger AuNP conjugate, **21a**, was obtained.

Purification process The deep red-colored solution of the crude complex was filtered using a Millipore Amicon Ultra-15 centrifugal filter (30 KMW cutoff) and the solid remaining on the filter redissolved for five cycles. Each time, the residue was dissolved in 15 mL of water/methanol mixture (70:30) and refiltered. After the fifth filtration cycle, the residue was dissolved in water and the solution filtered for four additional cycles with a 50 KMW cutoff filter. Then, the residue retained on the filter was dissolved in 10 mL water and extracted five times with CHCl_3 (15 mL each

time). The solvent was removed from the last CHCl_3 extract, and the organic phase residue was examined by mass spectroscopy to confirm that no small MW contaminants remained at detectable levels. After the final extraction step, the complex in the aqueous phase was lyophilized to obtain **21a**, AuNP conjugate (larger diameter) containing an AR agonist and a carboxylate-solubilizing moiety, as a brown/black-colored residue.

AuNP conjugate 21b

Synthesis Compounds **13** (2.51 mg, 2.12 μmol) and **16** (1.57 mg, 2.13 μmol) and DMF (3 mL) were added to a 25-mL round bottom flask. Hydrogen tetrachloroaurate(III) tetrahydrate (4.62 mg, 3 equiv., 12.2 μmol) was dissolved in 0.380 mL of water and added to the above flask, and the mixture was stirred for 30 min at room temperature. Sodium tetrahydroborate (1.37 mg, 36.1 μmol) in 2.25 mL water was added to the mixture dropwise. The pale yellowish color of the reaction mixture turned red immediately and was stirred for 4 h at room temperature. The solvent was evaporated at $\sim 40^\circ\text{C}$, and the residue containing **21b**, AuNP conjugate (smaller diameter) containing an AR agonist, and a carboxylate-solubilizing moiety, was redissolved in 15 mL of water.

Purification process The deep red-colored solution of the crude complex was filtered using a Millipore Amicon Ultra-15 centrifugal filter (30 KMW cutoff) and the solid remaining on the filter redissolved for five cycles. Each time, the residue was dissolved in 15 mL of water and refiltered. After the fifth filtration cycle, the residue was dissolved in water, and the solution was lyophilized. The lyophilized solid was dissolved in 1 mL of water and treated with 6 mL of THF, and the colloidal solution was centrifuged at $4,000\times g$ for 8 min to precipitate the AuNPs. This cycle of dissolving the residue in water followed by the addition of THF and finally centrifugation was repeated three times. The final residue was dissolved in water (2 mL) and transferred to a pre-weighed glass vial and lyophilized to obtain **21b**, as a brown/black-colored residue. Subsequent bioassays of supernatant fractions from this procedure demonstrated that they were free of small MW contaminants binding to ARs.

General procedure for the synthesis of AuNP conjugate 22

Synthesis Compounds **14** (2.10 mg, 1.06 μmol) and **16** (1.25 mg, 1.02 μmol) and DMF (2.4 mL) were added with stirring to a 25-mL round bottom flask. Hydrogen tetrachloroaurate(III) tetrahydrate (3.74 mg, 3 equiv., 9.04 μmol) was dissolved in 0.304 mL of water, and the solution was added to the above flask while stirring continuously for 30 min at room temperature. A solution of

sodium tetrahydroborate (1.08 mg, 28.6 μmol) in 1.8 mL water was added to the mixture dropwise. The pale yellowish color of the reaction mixture turned red immediately and was stirred for 4 h at room temperature. The solvent was evaporated, and the residue containing **22**, a xanthine AR antagonist AuNP conjugate containing a carboxylate-solubilizing moiety, was redissolved in 15 mL of a water/methanol mixture (75:25).

Purification process The deep red-colored complex was filtered using Millipore Amicon Ultra-15 centrifugal filter (30 KMW cutoff) five times in succession. Each time, the residue was dissolved in 15 mL of water/methanol mixture (70:30). After the fifth time, the residue was dissolved in water and filtered four times with a 50 KMW cutoff filter. The residue was dissolved in 10 mL water and extracted five times with CHCl_3 (15 mL). The solvent was removed from the final CHCl_3 extract, which was subjected to mass spectroscopy and a competitive binding assay at the $\text{A}_{2\text{A}}\text{AR}$ to confirm that no starting material was left in the complex. After the final extraction step, the complex was lyophilized to obtain **22** as a brown/black-colored residue.

General procedure for the synthesis of AuNP conjugates 23 and 24

Synthesis A mixture of compound **16** (1.80 mg, 2.01 μmol) and either **17** (2.36 mg, 2.02 μmol) or **18** (2.51 mg, 2.06 μmol) in DMF (23 mL) was added to a 100-mL round bottom flask. Hydrogen tetrachloroaurate(III) tetrahydrate (30.7 mg, 20 equiv., 81.0 μmol) dissolved in 2.46 mL of water was added to the above flask, and the mixture was stirred 30–45 min at room temperature. Sodium tetrahydroborate (9.12 mg, 242 μmol) in 14.7 mL water was added to the mixture slowly (using a dropping funnel over 30 min). The pale yellowish color of the reaction mixture turned deep red immediately, and the mixture was stirred for 4 h at room temperature. The solvent was evaporated, and the residue was dialyzed against water for 24 h, changing the water hourly. This residue contained either **23**, a nucleoside AR agonist AuNP conjugate containing a carboxylate-solubilizing moiety, or **24**, an $\text{A}_{2\text{A}}\text{AR}$ antagonist AuNP conjugate containing a carboxylate-solubilizing moiety.

Purification process The deep red-colored complex was filtered using Millipore Amicon Ultra-15 centrifugal filter (30 KMW cutoff) five times in succession. Each time, the residue was dissolved in 15 mL of water/methanol mixture (70:30). After the fifth time, the residue was dissolved in water and filtered four times with a 50 KMW cutoff filter. The residue was then dissolved in 10 mL of water and extracted with CHCl_3 (20 mL each, five times) until no

starting material remained in the organic phase (each organic fraction was analyzed using mass spectroscopy), and aqueous phase was lyophilized to obtain **23** and **24**.

AuNP conjugate 26a

Synthesis Compounds **14** (2.51 mg, 2.01 μmol) and **20** (1.80 mg, 2.02 μmol) and DMF (23 mL) were added to a 100-mL round bottom flask. Hydrogen tetrachloroaurate(III) tetrahydrate (30.7 mg, 20 equiv., 80.2 μmol) dissolved in 2.46 mL of water, and the solution added to the above flask. The pale yellow mixture was stirred for 30–45 min at room temperature. A solution of sodium tetrahydroborate (9.02 mg, 0.24 mmol) in 14.7 mL water was added slowly to the mixture (using a dropping funnel over 30 min), and the color changed from pale yellow to deep red indicating the formation of the AuNP conjugate. The solvent was evaporated, and the residue was dialyzed against water for 24 h, changing the water hourly, to obtain **26a**, a xanthine AR antagonist AuNP conjugate containing a phosphate-solubilizing moiety (larger diameter).

AuNP conjugate 26b

Synthesis Compounds **14** (2.51 mg, 2.01 μmol) and **20** (1.80 mg, 2.02 μmol) and DMF (6 mL) were added to a 100-mL round bottom flask. Hydrogen tetrachloroaurate(III) tetrahydrate (9.22 mg, 6 equiv. 24.2 μmol) was dissolved in 0.76 mL of water, the solution was added to the above flask, and the mixture stirred for 30–45 min at room temperature. Sodium tetrahydroborate (2.74 mg, 72.0 μmol) in 4.5 mL water was added slowly (during 30 min) to the mixture, using a dropping funnel. The pale yellowish color of the reaction mixture turned deep red immediately, and the mixture was stirred for 4 h at room temperature. The solvent was evaporated, and the residue was dialyzed against water for 24 h, changing the water hourly, to obtain **26b**, a xanthine AR antagonist AuNP conjugate containing a phosphate-solubilizing moiety (smaller diameter).

Purification process Each residue was then dissolved in 10 mL of water and extracted with CHCl_3 (20 mL each, five times), until no starting material remained in the organic phase (each organic fraction was analyzed successively using mass spectroscopy), and the aqueous phase was lyophilized to obtain compounds **26a** and **26b**.

Ultrapurification method

Solutions (4 μM) of the AuNP conjugates were prepared in 50 mM Tris–HCl (pH 7.5) and 10 mM MgCl_2 buffer. Wash buffer solutions, whose concentrations of Tris–HCl, MgCl_2 , and DMSO matched those of the solutions of AuNPs, were also prepared for rinsing the centrifugal filters prior to

ultrafiltration. Centrifugal filters (Millipore Ltd., Amicon Ultra-0.5, 10 KMW cutoff) were rinsed with 400 μL of wash buffer for 5 min at $14,000\times g$, after which the filter units were placed into fresh microcentrifuge tubes.

Ultrafiltration of the colloidal solutions of AuNPs was then performed by adding 400 μL of each AuNP solution to a filter assembly and then spinning the filters for 15 min at $14,000\times g$. The filtrate was transferred to a fresh microcentrifuge tube for analysis in a radioligand binding assay, and the solid material remaining on each filter was resuspended in 400 μL of buffer. The resuspended solution of AuNPs was collected by inverting and transferring the contents of the filter assembly to a fresh microcentrifuge tube and spinning the colloidal solution for 2 min at $1,000\times g$ to precipitate the larger particles that would interfere in the assay. Once collected, the remaining supernatant from each AuNP sample (approx. 20 μL) was diluted in a total of 400 μL of 50 mM Tris–HCl (pH 7.5) buffer containing 10 mM MgCl_2 . Finally, the fractions collected from the initial filter rinse and the ultrafiltration steps and the resuspended AuNPs were tested in a radioligand binding assay, as described below.

Calculation of the number of ligands per AuNP using NMR studies

NMR spectra of the AuNP conjugates (2–8 mg) were measured in D_2O (300 μL). After that, to the same NMR tube, a known amount of DMF was added as an internal standard and the NMR peak intensities of the DMF formyl proton at 8.04 ppm and PEG methylene groups at 3.62 ppm were compared. The ratio was used to calculate the number of PEG-containing groups on average attached to each AuNP (see [Supporting Information](#)).

Assessment by analytical HPLC of the stability of the monomeric arms containing AR ligands (**14**, **18**) in the presence of either CHO or human embryonic kidney 293 cell membranes

Assay solutions containing a monomeric arm (diluted from a 5-mM DMSO stock solution of either **14** or **18**, 10 μM final concentration) and either Chinese hamster ovary (CHO) or human embryonic kidney 293 (HEK293) cell membrane suspensions (100 $\mu\text{g}/\text{mL}$) were prepared in 50 mM Tris–HCl (pH 7.5) and 10 mM MgCl_2 buffer. The solutions in glass culture tubes (20 $\times$ 75 mm) were incubated at 25 $^\circ\text{C}$ for 1 h in a shaking water bath. Then, the solutions were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 15 min at $14,000\times g$ to separate the cell membrane fragments from the solution. The supernatants of the solutions were then analyzed for degradation of the monomeric arms using an analytical Hewlett-Packard 1100 HPLC

equipped with a Zorbax Eclipse 5- μ m XDB-C18 analytical column (250 $\times$ 4.6 mm; Agilent Technologies Inc, Palo Alto, CA, USA). Mobile phase: linear gradient solvent system: 5 mM TBAP-CH₃CN from 80:20 to 40:60 in 6.5 min; the flow rate was 1 mL/min system. Peaks (retention time was 3.22 min for **14** and 7.62 min for **18**) were detected by UV absorption with a diode array detector at 254, 275, and 280 nm, and AUC was calculated based on the peak at 254 nm.

Competitive radioligand binding assays at ARs

Competition radioligand binding experiments were conducted to determine the binding affinities of conjugated AuNPs (see [Supporting Information](#)). A range of concentrations of either AuNPs or monomeric arms between 0.1 nM and 10 μ M was tested in competing for binding to human (h) A₃ or A_{2A}ARs on cell membranes derived from A₃AR-expressing CHO cells or A_{2A}AR-expressing HEK293 cells. The radioligands used were [¹²⁵I]I-AB-MECA (*N*⁶-(4-amino-3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine) and [³H]CGS21680 (2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5'-*N*-ethylcarboxamidoadenosine) for A₃AR and A_{2A}AR binding, respectively, by methods described [17]. Selected compounds were tested at the hA₁AR using A₁AR-expressing CHO cells and [³H]PIA (*N*⁶-*R*-phenylisopropyladenosine) as the radioligand [17]. For some of the larger AuNP derivatives, an increase in radioactivity bound to the glass fiber filter was observed with increasing concentrations of AuNPs, which precluded determination of a binding curve.

Assessment by radioligand binding of the stability of a representative AuNP conjugate (**26a**) in the presence of HEK293 cell membranes

A solution containing compound **26a**, AR antagonist AuNP conjugate containing a phosphate-solubilizing group, from an H₂O stock solution (1 μ M, final volume 250 μ M) and membranes of control HEK293 cells not expressing an AR (100 μ g/mL) was prepared in 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂ buffer (binding buffer). In addition, a 250- μ L solution containing only control HEK293 cell membranes and another one containing control HEK293 cell membranes in the presence of 10 μ M nonselective agonist 5'-*N*-ethylcarboxamidoadenosine (NECA) were prepared. These solutions in glass culture tubes (20 $\times$ 75 mm) were incubated for 1 h at 25 °C in a shaking water bath. Then, the solutions were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 20 min at 14,000 $\times$ *g* to pellet the cell membrane fragments from the solutions. The supernatants of the solutions were run through centrifugal filter units (Millipore Ltd., Amicon Ultra-0.5, 10 KMW cutoff) to collect filtrates (approximate volume of each filtrate 220 μ L). Then, the material remaining on the centrifugal filter

units (approximate volume 30 μ L) was collected and resuspended in binding buffer to the starting volume (250 μ L). Then, the filtrates and recovered materials were each tested for binding activity in a radioligand binding assay using cell membranes from HEK293 cells stably expressing the hA_{2A}AR, as described above.

Results

Design and chemical synthesis

We have explored strategies for conjugating small molecular ligands for ARs to AuNPs with retention of biological activity. A₃AR agonists **1** and **2** have shown similar hA₃AR binding affinities (*K*_i~1 nM) and have been used widely as pharmacological probes [13]. A ring-constrained North (N)-methanocarpa derivative of **2** was recently reported to be a highly selective A₃AR agonist, MRS3558 (**3**), having a *K*_i value in binding to the hA₃AR of 0.3 nM [12]. Tosh et al. modified **3** at the adenine C2 position with a dialkyne to form **4** that retained hA₃AR affinity (*K*_i=23 nM) and was suitable for linking at the terminal alkyne with an azide through a 2+3 cycloaddition (click) reaction [17]. We also used a moderately potent and selective A_{2A}AR agonist, CGS21680 (**5**), a C2 derivatized adenosine 5'-carboxamide, for immobilization on AuNPs through condensing its terminal carboxylic acid with an amine. Nevertheless, it is recognized that the A_{2A}AR selectivity may be limited because the hA₃AR affinity of this nucleoside is substantial. Selective A_{2A}AR antagonists, pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine-5-amine (PTP) derivatives, were explored for chain functionalization to arrive at amine functionalized congener **6** to provide a means of covalent attachment by amide formation of sterically bulky groups without losing the ability to bind to the A_{2A}AR with high affinity [21]. The structure-activity relationship of xanthine derivatives as AR antagonists has been exhaustively explored [22]. Heptynoyl-XAC (**7**), an analogue of the 8-phenyl derivative xanthine amine congener (XAC), is relatively potent but nonselective in binding to hARs [19]. This terminal alkyne can be reacted with azides by click chemistry with retention of AR affinity, as was shown previously with dendrimer conjugates [19].

In Fig. 2, we describe the synthesis of PEGylated derivatives of a selective A₃AR agonist **13** and nonselective AR antagonist **14**, for AuNP conjugation from a commercially available polyethylene glycol (PEG) azide **11** and TA **10** via amide coupling to obtain **12** [18, 23]. The key triazole compounds, **13** and **14**, were obtained after a click reaction of azide **12** and an alkyne, e.g., **4** or **7** [17].

PEGylated derivatives of the selective A_{2A}AR agonist **5** and antagonist **6**, armed with TA for AuNP conjugation (compounds **17** and **18**, respectively) were synthesized

Fig. 2 Functionalization of A₃AR agonist **4** and AR antagonist **7** for coupling to AuNPs. Reagents and conditions: *a* DCC, DMAP, DCM, 0 °C, 2 h, rt, overnight; *b* (1 eq) sodium ascorbate, (0.5 eq) CuI, H₂O/DMF (1:3), TBTA, rt, overnight

according to Fig. 3. The PEGylated azide **12** was reduced to its amine **15** using a Staudinger reaction, followed by coupling with succinic anhydride in pyridine to provide the carboxyl-terminated compound **16** [18]. The coupling of compounds **5** with **15** and **6** with **16** using PyBOP and DIEA gave the desired intermediates **17** and **18**.

Initial preparation of AuNPs coated with our agonists/antagonists alone resulted in particles with low aqueous solubility. This prompted the use of solubilizing “doping” agents in the nanoparticle synthesis. An obvious choice was the carboxylic acid-terminated linker **16** (previously synthesized in Fig. 3). In addition, we prepared the monophosphoryl linker **20** for increasing aqueous solubility from azide **12** and alkynyl phosphate **19** using click chemistry (Fig. 4). Both molecules **16** and **20** alone did not show any AR binding activity.

The synthesis of AuNPs coated with AR ligands **13**, **14**, **17**, and **18** along with solubilizing carboxylate chain **16** is shown in Fig. 5. Alternately, ligand **14** was also coated on AuNPs that were doped with solubilizing monophosphate **20** (Fig. 6). The diameters of the AuNPs were 1.8–4.5 nm, as shown by transmission electron microscopy (Fig. 7a–f). This size range was chosen because it was small enough for imaging purposes, and similarly sized AuNPs have shown slower kinetics of cellular uptake than larger particles [24]. The anchor point of the tethered GPCR ligands consisted of a TA moiety containing two thiol groups to achieve a relatively stable bond with the nanoparticle [37]. A segment of ten PEG units attached to the carboxylic group of the thioctic acid contributed to water solubility and was intended to allow the interaction of the immobilized GPCR

ligand with the adaptable binding site of the GPCR. In a typical reaction, an equimolar amount of the AR ligand and either carboxylate **16** or monophosphate **20** and 3 M equivalents of tetrachloroauric(III) acid (HAuCl₄·3H₂O) in DMF were reduced with 6 M equivalents of NaBH₄ [10]. Using this synthetic approach, we expected (a) to achieve the ability to functionalize the AuNPs with multiple ligands, (b) to increase the solubility of the AuNPs by attaching a water-solubilizing ligand, and (c) to favorably alter the AuNP surface with anionic ligands containing COOH groups to lower the cellular uptake of the particles.

The GPCR ligand-conjugated AuNPs were characterized using UV–visible absorbance spectroscopy, TEM, and NMR. Figure 7g shows the UV–visible absorption spectra collected from a set of AuNP samples with different tethered ligands; some representative TEM images collected from these samples are also shown. The absorption spectra show that at low Au-to-ligand (smaller size NPs) molar ratios, the characteristic shape-dependent surface plasmon absorption band (SPB) located at 520 nm is weak (**22** (2.5 nm diameter) and **23** (3 nm)). However, the well-defined and narrow SPB peaks at higher Au/ligand molar ratios (relatively larger NPs (**24** (4.2 nm) and **26a** (4.5 nm))) indicated a narrow size distribution of these AuNPs. DLS studies confirmed that **24** and **26a** have a larger diameter and a narrow size distribution (Fig. 7h).

The stoichiometry of substitution was determined using an NMR method [42] and compared to the calculated substitution based on reactants. The AuNPs were examined for the presence of noncovalently attached small molecules that could complicate the biological characterization by leaching

Fig. 3 Functionalization of A_{2A} AR agonist **5** and antagonist **6** for coupling to AuNPs. Reagents and conditions: *a* PPh_3 , THF, H_2O ; *b* succinic anhydride, pyridine; *c* PyBOP, DIEA, DMF, overnight

from the particles. These noncovalently attached small molecules were removed via filtration and/or extraction techniques. For several conjugates, because of complications in the binding assay, it was necessary to prepare two sizes of AuNP conjugates, i.e., both large diameter (**21a** and **26a**, 4–5 nm) and smaller diameter (**21b** and **26b**, ~2 nm).

Pharmacological characterization

AR ligand-bearing AuNPs were characterized pharmacologically for their binding affinities and functional effects in mammalian cells expressing three subtypes of hARs. Radioligand binding assays were carried out using standard 3H and ^{125}I -labeled nucleosides (Table 1) [17, 21]. The membrane preparations were prepared from CHO cells (A_1 and A_3) or HEK293 cells (A_{2A}) stably expressing a hAR subtype. The solubilizing moieties alone **16**, **20** or as an AuNP conjugate **25**, **27** did not bind appreciably to ARs. Before testing the AuNP conjugates, we ascertained that low MW components of significant biological activity (i.e., that passed through ultrafiltration with 10–30 kDa cutoff) were not

present. Also, the stability of the monomeric ligands with attached tether (**14**, **18**) in the presence of HEK293 cell membranes was demonstrated (Supporting Information).

The A_3 AR affinity of 2-alkynyl adenosine conjugate **21b** of smaller diameter (K_i 14.8 nM) was increased by twofold in comparison to the monomeric agonist ligand **13**. A K_i value for the corresponding large diameter particle **21a** at the A_3 AR could not be obtained because of an unexpected increase of radioligand bound, rather than inhibition. The A_{2A} AR affinity of monomeric PTP antagonist derivative **18** was also well preserved in the corresponding AuNP conjugate **24** (K_i 34.9 nM). Binding of **24** at the A_1 AR was also shown. However, the moderate A_{2A} AR affinity (K_i 1.42 μ M) of XAC monomeric antagonist derivative **14** was not maintained in the AuNP conjugate **22** containing carboxylate water-solubilizing groups, but the A_3 AR affinity of **22** (K_i 262 nM) was 12-fold greater than the monomeric precursor **14**. The XAC conjugate containing phosphate water-solubilizing groups **26a** (large diameter) did bind potently to both A_1 (K_i 211 nM) and A_{2A} ARs (K_i 69 nM). A K_i value for this large diameter particle **26a** at the A_3 AR could not be obtained because

Fig. 4 Synthesis of a water-solubilizing ligand. Reagents and conditions: *a* sodium ascorbate (1 eq), $CuSO_4$, (0.5 eq), H_2O/DMF (1:3), rt, overnight

Fig. 5 Coupling of AR agonists **13** and **17** and antagonists **14** and **18** to AuNPs. For the A₃AR agonist, both large diameter **21a** (4–5 nm) and smaller diameter **21b** (~2 nm) conjugates were prepared. Reagents and conditions: *a* HAuCl₄, NaBH₄, DMF

of increased radioligand bound. The smaller diameter AuNP conjugate **26b** was shown to bind to both A_{2A} and A₃ARs with submicromolar K_i values. Thus, the phosphate water-solubilizing moiety was advantageous for retaining receptor recognition. The A_{2A}AR agonist conjugate **23** was inactive in binding to that receptor. Similarly linked derivatives of **17** on quantum dots also failed to bind appreciably to the A_{2A}AR [23], suggesting that there might be differences in the tolerance of the various AR subtypes for solid particle attachment. However, **23** bound to the A₃AR with a K_i of 240 nM, suggesting a difference between the A_{2A}AR and A₃AR structures in the steric restrictions for binding conjugates.

Functional data were determined in an assay consisting of hA₃AR-induced inhibition of the production of cAMP in membranes of CHO cells expressing the hA₃AR [38]. The

C2 alkynyl AuNP conjugate **21b** was a full agonist at the A₃AR, showing a relative efficacy of 100 % (relative to inhibition by 10 μ M NECA). A full concentration response curve in hA₃AR-mediated inhibition of adenylyate cyclase provided an EC₅₀ value of 13.6 nM (Fig. 8), i.e., identical to its binding affinity.

Discussion

GPCRs are a superfamily of cell surface signaling receptors having important roles in many physiological functions and disease states, including cancer and cardiovascular disease [12]. Many of the currently approved drugs that target GPCRs achieve excellent therapeutic benefits. Therefore, the

Fig. 6 Coupling of AR antagonist **14** to AuNPs. Reagents and conditions: *a* HAuCl₄, NaBH₄, DMF

development of new GPCR-based drugs is a well-validated and ongoing process in the development of therapies for many diseases including cancer [11]. There is increasing interest in the therapeutic potential of selective AR agonists/antagonists for treating a wide range of diseases including cancer.

The ARs are overexpressed in various tumor cells, and their activation by certain selective agonist ligands has been shown to inhibit tumor growth via a range of signaling pathways. The A₃AR can be found in different types of tumor cells such as K562 human leukemia, Jurkat lymphoma, U937 human monocytic/macrophagic cells, Nb2 rat lymphoma, A375 human melanoma, PGT-beta mouse pineal gland tumor cells, human glioblastoma, and human prostatic cells [26–29]. The A₃AR expression level is high in human melanoma, colon, breast, small cell lung, and pancreatic carcinoma as compared to normal tissue, suggesting upregulation of the A₃AR in cancer growth. The A₃AR agonists were found to inhibit growth of various tumors in vivo. These findings of A₃AR overexpression in different tumor cell types suggest that this receptor may be a specific target in novel cancer treatment.

The A_{2A}AR is expressed on many lymphoid cells, including neutrophils, monocytes, macrophages, T cells, and natural killer cells [13]. Activation of this receptor by adenosine or adenosine analogues (A_{2A}AR agonist) results in a wide range of anti-inflammatory and immunosuppressive responses [12, 13]. In vitro studies showed A_{2A}AR agonists to protect against excessive inflammation and tissue injury in the liver, heart, kidney, and vasculature. The A_{2A}AR is overexpressed in the striatum and olfactory tubercle of the brain. The A_{2A}AR is coupled to stimulation of adenylyl cyclase, and its antagonism mediates many of the CNS stimulatory effects of caffeine in humans [13]. Other potential therapeutic applications of selective A_{2A}AR agonists are as anti-aggregatory, antipsychotic, and anti-Huntington's disease agents.

Nanoparticles such as AuNPs could offer many advantages in the development of therapeutics due to their multivalent binding abilities and biocompatibility. Toxicity of AuNPs depends on their size, shape, surface functionality, surface charge, and hydrodynamic radius [39, 40]. The low in vivo toxicity of AuNPs, especially of relatively small diameter, has been described [39]. Although many in vitro toxicity studies of various AuNPs have been done, more in vivo studies are needed [40]. We envision the use of these AuNP conjugates for both diagnostic and therapeutic purposes. The presence of the attached Au particle could affect the action of the drug molecules by altering pharmacokinetic or biodistribution properties. The nanometer size of these particles may either help to avoid penetration of the tethered drug to certain tissues in the body or it may enhance the exposure of larger particles to certain tissues, such as tumors by the enhanced permeability and retention (EPR) effect [1]. Targeting extracellular GPCRs that are known to be overexpressed in tumor cells, such as ARs, with ligand-conjugated NPs may have an added advantage for targeted therapy over the EPR effect alone. Many studies have shown that attaching a targeted ligand to NPs enhances their biodistribution within solid tumors [30, 31]. Thus, tethering an AR ligand that may have its own anticancer activity, such as an A₃AR agonist or A_{2A}AR antagonist [32], could serve both targeting and therapeutic purposes. The methodology introduced in this study for AR agonists and antagonists could potentially be applied to other GPCR ligands.

Moreover, AuNPs have been extensively developed for selectively inducing hyperthermia for localized ablation of cancerous tumors. Thus, AuNPs are delivered to a tumor either by active or passive targeting, and the tumor then is exposed to the radiation such as NIR laser light, radiowaves, or an alternating magnetic field [33–36]. Another very

Fig. 7 Characterization of AuNP conjugates: TEM images (average AuNP diameter) of **a** **21b**. Scale bar = 10 nm (2.00 ± 0.07 nm). **b** **22**. Scale bar = 20 nm (2.56 ± 0.09 nm). **c** **23**. Scale bar = 20 nm (3.0 ± 0.12 nm). **d** **24**. Scale bar = 20 nm (4.24 ± 0.10 nm). **e** **26a**. Scale bar = 20 nm (4.51 ± 0.07 nm). **f** **26b**. Scale bar = 10 nm (1.89 ± 0.12 nm). **g** UV–visible absorption spectra for AuNP conjugates in H_2O , and **h** DLS profiles of **24**, **26a**

important property of AuNPs is that they can be used as imaging agents for cancer detection. The versatile optical properties of AuNPs have enabled optical imaging of cells with a wide variety of contrast mechanisms.

In the present study, we have demonstrated the feasibility of coupling functionalized small GPCR ligands to AuNPs with retention of high affinity binding to the receptor. As a first example of this approach, we selected agonist and

Table 1 AR binding affinity of AuNP conjugates and intermediates

Compound	A _{2A} AR, K _i (nM) or % inhibition, 1 μ M	A ₃ AR, K _i (nM) or % inhibition, 1 μ M	Average no. of ligands per AuNP
AR ligand monomers			
13	ND	37.4 $\pm$ 9.0	N/A
14	1,420 $\pm$ 570	3,040 $\pm$ 1,050	N/A
17	44.0 $\pm$ 3.0	245 $\pm$ 18	N/A
18	61.0 $\pm$ 3.0	4,060 $\pm$ 830	N/A
Au conjugates of AR ligands			
21a	ND	ND ^a	600
21b	ND	14.8 $\pm$ 1.8	42
22	21 %	262 $\pm$ 29	36
23	28 %	240 $\pm$ 39	36
24	34.9 $\pm$ 3.5	ND	140
26a	69.0 $\pm$ 9.0	ND ^a	770
26b	370 $\pm$ 40	750 $\pm$ 120	20
Solubilizing moieties and Au conjugates			
16	0 %	0 %	N/A
20	0 %	0 %	N/A
25	30 %	0 %	N/A
27	28 %	ND ^a	N/A

Using standard radioligands (A₁: [³H]N⁶-R-phenylisopropyladenosine, A_{2A}: [³H]2-[p-(2-carboxyethyl)-phenylethylamino]-5'-N-ethylcarboxamido-adenosine, or A₃: [¹²⁵I]N⁶-(4-amino-3-iodobenzyl)adenosine-5'-N-methyl-uronamide) [17, 25]. Values shown as mean $\pm$ SEM were for three determinations done in duplicate. The concentration of the AuNP was used in the calculation, not the concentration of individual pharmacophoric units. Three compounds were tested at A₁ (K_i, nM): **24**, 211; **26a**, 211; **18**, 35 % inhibition at 10 μ M

ND not determined, N/A not applicable

^a Total binding in the A₃AR assay increased with increasing concentration of ligand for **21a** and **26a** (larger diameter particles) likely because of trapping of the AuNPs in the filter, but this phenomenon did not occur with **21b** and **26b** (smaller diameter particles)

Fig. 8 Functional agonism by **21b** in an assay of adenylate cyclase in CHO cells expressing the hA₃AR (EC₅₀ value 13.6 nM). The activity of 10 μ M 5'-N-ethyluronamidoadenosine represented 100 % efficacy. The experiment was repeated three times, and the average is shown

antagonist ligands for A_{2A} and A₃ARs. We functionalized AuNPs by tethering specific ligands, agonists and antagonists, of ARs to the gold surface as models for cell surface interactions with GPCRs. The AuNP conjugates with chain-extended AR ligands alone (PEGylated nucleosides and nonnucleosides, anchored to the Au via thioctic acid) were found insoluble in water due to hydrophobic entities in the ligand. Therefore, we added a second, biologically inactive pendant moiety, to increase the water solubility, consisting of a PEGylated chain terminating in a carboxylic or phosphate group. Pharmacological receptor binding studies on these GPCR ligand-derivatized AuNPs (2–5 nm in diameter) performed using membranes of mammalian cells stably expressing hA₁, A_{2A}, and A₃ARs, showed that the desired selectivity was retained with K_i values (nanomolar) of A₃AR agonist **21b** and A_{2A}AR antagonists **24** and **26a** of 14 (A₃), 34 (A_{2A}), and 69 (A_{2A}), respectively. The corresponding monomers displayed K_i values of 37, 61, and 1,420 nM, respectively. Thus, the A_{2A}AR affinity of the antagonist conjugate **26a** was 21-fold greater than the xanthine monomer **14**. The monomer **18** of antagonist conjugate **24** was 67-fold selective for the A_{2A}AR in comparison to the A₃AR, and its AuNP conjugate had 2-fold greater affinity than the monomer. Also, the nucleoside conjugate **21b** potentially activated the A₃AR.

Several parallel synthetic approaches were used to prepare new PEGylated derivatives via conjugating the above AR ligands to TA-PEG-N₃ **12**, TA-PEG-NH₂ **15**, or TA-PEG-NHCO(CH₂)₂COOH **16** with click or amide coupling chemistry. TA groups and PEG chains were known to increase the water solubility, but AuNP conjugates of a PEGylated adenosine derivative were found insoluble in water due to aromatic and aliphatic entities in the ligand. Addition of a second, negatively charged ligand (TA-PEG-NHCO(CH₂)₂COOH **16** or monophosphate tether **20**) together with the PEGylated AR ligand increased the nanoparticle water solubility and allowed biological evaluation. TEM images revealed that the gold core had an average diameter of ~2 nm for complex **21b** (Fig. 7a). The diameter of the AuNPs could be reliably controlled by the initial molar ratio of Au to the combination of AR ligand monomer and solubilizing moiety, if present, during the preparation. This ratio varied from 3 (e.g., smaller particle **21b**) to 20 (e.g., larger particle **21a**) [41].

Purification of multivalent AuNP conjugates was very challenging because one is limited mainly to dialysis, filtration, or centrifugation. Additionally, the effect from untethered/trapped small molecules on the AuNP conjugates can affect the biological results. We found that purification of AuNP conjugates via dialysis and/or filtration did not eliminate all small MW contamination. However, extraction of the AuNPs with CHCl₃ or THF (for **21b**) was sufficient to remove such contaminants prior to biological evaluation.

In conclusion, we have introduced a strategy for applying the potential of nanotechnology for targeted drug delivery for GPCRs. These encouraging initial results—that GPCR affinity can be maintained in AuNP conjugates with appropriate functionalization—need to be followed by in vivo pharmacokinetic studies. By combining nanomedicine approaches based on AuNPs targeting specific GPCRs that are associated with or are known to be dysregulated in disease states, it may be possible to enhance the functionality of such conjugates over the parent small molecule GPCR ligands. Although we have demonstrated the feasibility of coupling small AR agonists and antagonists to AuNPs, in some cases with retention of high affinity and/or selectivity, this strategy could become a general approach for a wide range of GPCR ligands. The feasibility of performing such imaging studies using AuNPs functionalized with ligands for ARs and other GPCRs can be carried out initially in in vitro studies. Such conjugates may eventually prove useful in the diagnosis or treatment of cancer and other diseases.

Acknowledgments We thank Dr. Noel Whittaker (NIDDK) for MS measurements. This research was supported by the Intramural Research Programs of NIDDK and NCI, National Institutes of Health.

References

- Arvizo R, Bhattacharya R, Mukherjee P (2010) Gold nanoparticles: opportunities in nanomedicine. *Expert Opin Drug Deliv* 7:753–763
- Chitrani DB (2010) Intracellular uptake, transport, and processing of gold nanostructures. *Mol Membr Biol* 27:299–311
- Huang X, Peng X, Wang Y, Shin DM, El-Sayed MA, Nie S (2010) A reexamination of active and passive tumor targeting by using rod-shaped gold nanocrystals and covalently conjugated peptide ligands. *ACS Nano* 4:5887–5896
- Khlebtsov N, Dykman L (2011) Biodistribution and toxicity of engineered gold nanoparticles: a review of in vitro and in vivo studies. *Chem Soc Rev* 40:1647–1671
- Han G, Ghosh P, Rotello VM (2007) Functionalized gold nanoparticles for drug delivery. *Nanomedicine (London)* 2:113–123
- Cai W, Gao T, Hong H, Sun J (2008) Applications of gold nanoparticles in cancer. *Nanotechnol Sci Appl* 1:17–32
- Boisselier E, Astruc D (2009) Gold nanoparticles in nanomedicine: preparations, imaging, diagnostics, therapies and toxicity. *Chem Soc Rev* 38:1759–1782
- Patel PC, Giljohann DA, Daniel WL, Zheng D, Prigodich AE, Mirkin CA (2010) Scavenger receptors mediate cellular uptake of polyvalent oligonucleotide-functionalized gold nanoparticles. *Bioconjug Chem* 21:2250–2256
- Bardhan R, Chen W, Perez-Torres C, Bartels M, Huschka RM, Zhao LL, Morosan E, Pautler RG, Joshi A, Halas NJ (2009) Nanoshells with targeted simultaneous enhancement of magnetic and optical imaging and photothermal therapeutic response. *Adv Funct Mater* 19:3901–3909
- Li L, Zhang Q, Liu A, Li X, Zhou H, Liu Y, Yan B (2011) Proteome interrogation using nanoprobe to identify targets of a cancer-killing molecule. *J Am Chem Soc* 133:6886–6889
- Lappano R, Maggiolini M (2011) G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov* 10:47–60
- Jacobson KA, Gao ZG (2006) Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* 5:247–264
- Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller C (2011) Nomenclature and classification of adenosine receptors—an update. *Pharmacol Rev* 63:1–34
- Madi L, Bar-Yehuda S, Barer F, Ardon E, Ochaion A, Fishman PA (2003) A₃ adenosine receptor activation in melanoma cells. *J Biol Chem* 278:42121–42130
- Yu L, Coelho JE, Zhang X, Fu Y, Tillman A, Karaoz U, Fredholm BB, Weng Z, Chen JF (2009) Uncovering multiple molecular targets for caffeine using a drug target validation strategy combining A_{2A} receptor knockout mice with microarray profiling. *Physiol Genomics* 37:199–210
- Smith NJ, Bennett KA, Milligan G (2011) When simple agonism is not enough: emerging modalities of GPCR ligands. *Mol Cell Endocrinol* 331:241–247
- Tosh DK, Chinn M, Yoo LS, Kang DW, Luecke H, Gao ZG, Jacobson KA (2010) 2-Dialkynyl derivatives of (N)-methanocarba nucleosides: “clickable” A₃ adenosine receptor-selective agonists. *Bioorg Med Chem* 18:508–517
- Susumu K, Uyeda HT, Medintz IL, Pons T, Delehanty JB, Mattoussi H (2007) Enhancing the stability and biological functionalities of quantum dots via compact multifunctional ligands. *J Am Chem Soc* 129:13987–13996
- Kecskés A, Tosh DK, Wei Q, Gao ZG, Jacobson KA (2011) GPCR ligand dendrimer (GLiDe) conjugates: adenosine receptor interactions of a series of multivalent xanthine antagonists. *Bioconjug Chem* 22:1115–1127
- Das A, Ko H, Burianek LE, Barrett MO, Harden TK, Jacobson KA (2010) Human P2Y₁₄ receptor agonists: truncation of the hexose moiety of uridine-5′-diphosphoglucose and its replacement with alkyl and aryl groups. *J Med Chem* 53:471–480
- Kumar TS, Mishra S, Deflorian F, Yoo SL, Phan K, Kecskés M, Szabo A, Shinkre B, Gao ZG, Trenkle W, Jacobson KA (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
- Baraldi PG, Tabrizi MA, Gessi S, Borea PA (2008) Adenosine receptor antagonists: translating medicinal chemistry and pharmacology into clinical utility. *Chem Rev* 108:238–263
- Das A, Sanjayan GJ, Kecskés M, Yoo L, Gao ZG, Jacobson KA (2010) Nucleoside conjugates of quantum dots for characterization of G protein-coupled receptors: strategies for immobilizing A_{2A} adenosine receptor agonists. *J Nanobiotechnol* 8:11–40
- Verma A, Stellacci F (2010) Effect of surface properties on nanoparticle–cell interactions. *Small* 6:12–21
- Hasko G, Linden J, Cronstein B, Pacher P (2008) Adenosine receptors: therapeutic prospects for inflammatory and immune diseases. *Nat Rev Drug Discov* 7:759–770
- Gessi S, Varani K, Merighi S, Cattabriga E, Iannotta V, Leung E, Baraldi PG, Borea PA (2002) A₃ adenosine receptors in human neutrophils and promyelocytic HL60 cells: a pharmacological and biochemical study. *Mol Pharmacol* 61:415–424
- Gessi S, Merighi S, Varani K, Borea PA (2011) Adenosine receptors in health and disease. *Adv Pharmacol* 61:41–75
- Jajoo S, Mukherjee D, Watabe K (2009) Adenosine A₃ receptor suppresses prostate cancer metastasis by inhibiting NADPH oxidase activity. *Neoplasia* 11:1132–1145
- Fishman P, Bar-Yehuda S, Synowitz M, Powell JD, Klotz KN, Gessi S, Borea PA (2009) Adenosine receptors and cancer. *Handb Exp Pharmacol* 193:399–441
- Pirollo KF, Chang EH (2008) Does a targeting ligand influence nanoparticle tumor localization or uptake? *Trends Biotechnol* 26:552–558

31. Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, Triche TJ (2005) Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res* 65:8984–8992
32. Ohta A, Kjaergaard J, Sharma S, Mohsin M, Goel N, Madasu M, Fradkov E, Sitkovsky M (2009) In vitro induction of T cells that are resistant to A₂ adenosine receptor-mediated immunosuppression. *Br J Pharmacol* 156:297–306
33. Kennedy LC, Bickford LR, Lewinski NA, Coughlin AJ, Hu Y, Day ES, West JL, Drezek RA (2011) A new era for cancer treatment: gold-nanoparticle-mediated thermal therapies. *Small* 7:169–183
34. Hirsch LR, Stafford RJ, Bankson JA, Sershen SR, Rivera B, Price RE, Hazle JD, Halas NJ, West JL (2003) Nanoshell-mediated near-infrared thermal therapy of tumors under magnetic resonance guidance. *Proc Natl Acad Sci U S A* 100:13549–13554
35. Gannon CJ, Patra CR, Bhattacharya R, Mukherjee P, Curley SA (2008) Intracellular gold nanoparticles enhance non-invasive radiofrequency thermal destruction of human gastrointestinal cancer cells. *J Nanobiotechnol* 6:2
36. Johannsen M, Gneveckow U, Eckelt L, Feussner A, Waldofner N, Scholz R, Deger S, Wust P, Loening SA, Jordan A (2005) Clinical hyperthermia of prostate cancer using magnetic nanoparticles: presentation of a new interstitial technique. *Int J Hyperthermia* 21:637–647
37. Henderson LC, Altimari JM, Dyson G, Servinis L, Niranjana B, Risbridger GP (2011) A comparative assessment of α -lipoic acid N-phenylamides as non-steroidal androgen receptor antagonists both on and off gold nanoparticles. *Bioorg Chem* 40:1–5
38. Nordstedt C, Fredholm BB (1990) A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal Biochem* 189:231–234
39. Longmire M, Choyke PL, Kobayashi H (2008) Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats. *Nanomedicine* 3:703–717
40. Aillon KL, Xie Y, El-Gendy N, Berkland CJ, Forrest ML (2009) Effects of nanomaterial physicochemical properties on in vivo toxicity. *Adv Drug Deliv Rev* 61:457–466
41. Oh E, Susumu K, Goswami R, Mattoussi H (2010) One-phase synthesis of water-soluble gold nanoparticles with control over size and surface functionalities. *Langmuir* 26:7604–7613
42. Manea F, Bindoli C, Polizzi S, Lay L, Scrimin P (2008) Expedient synthesis of water-soluble, monolayer-protected gold nanoparticles of controlled size and monolayer composition. *Langmuir* 24:4120–4124
43. Cohen S, Stemmer SM, Zozulya G, Ochaion, Patoka R, Barer F, Bar-Yehuda S, Rath-Wolfson L, Jacobson KA, Fishman P. (2011) CF102 an A₃ adenosine receptor agonist mediates anti-tumor and antiinflammatory effects in the liver. *J Cell Physiol* 226:2438–2447