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Novel Fluorescent Antagonist as a Molecular Probe in A₃ Adenosine Receptor Binding Assays Using Flow Cytometry

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Abstract

The physiological role of the A₃ adenosine receptor (AR) was explored in cardiac ischaemia, inflammatory diseases and cancer. We report a new fluorophore-conjugated human (h) A₃AR antagonist for application to cell-based assays in ligand discovery and for receptor imaging. Fluorescent pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-ylamine (pyrazolo-triazolo-pyrimidine, PTP) and triazolo[1,5-c]quinazolin-5-ylamine (triazolo-quinazoline, TQ) AR antagonists were compared. A chain-extended and click-conjugated Alexa Fluor-488 TQ derivative (MRS5449) displayed a radioligand binding K_i value of 6.4 ± 2.5 nM in hA₃AR-expressing CHO cell membranes. MRS5449 antagonized hA₃AR agonist-induced inhibition of cyclic AMP accumulation in a concentration-dependent manner (K_B 4.8 nM). Using flow cytometry (FCM), MRS5449 saturated hA₃ARs with very high specific-to-nonspecific binding ratio with an equilibrium binding constant 5.15 nM, comparable to the K_d value of 6.65 nM calculated from kinetic experiments. K_i values of known AR antagonists in inhibition of MRS5449 binding in whole cell FCM were consistent with radioligand binding in membranes, but agonist binding was 5–20 fold weaker than obtained with agonist radioligand [¹²⁵I]-AB-MECA. Further binding analysis of MRS5449 suggested multiple agonist binding states of the A₃AR. Molecular docking predicted binding modes of these fluorescent antagonists. Thus, MRS5449 is a useful tool for hA₃AR characterization.

Keywords

purines; fluorescence; G protein-coupled receptor; A₃ adenosine receptor; flow cytometry

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1. Introduction

Activation of the G_i protein-coupled A_3 adenosine receptor (AR) is associated with anticancer, antiischemic and antiinflammatory effects, and clinical trials of several prototypical selective agonists are underway [1,2]. A_3 AR antagonists are being examined as promising agents for the treatment of glaucoma [3,4]. Characterization of the A_3 AR in tissues and assays to establish a structure-activity relationship (SAR) of newly synthesized compounds for drug discovery, as for many other G protein-coupled receptors (GPCRs), is often dependent on the use of high affinity radioligands [5]. Fluorescent agonists and antagonists of GPCRs have been studied as molecular probes for binding experiments [6–8]. Recently, several 4,4-difluoro-4-bora-3a,4a-diaza-sindacene (BODIPY) derivatives have been reported as fluorescent ligands of the ARs, including the A_3 AR [9,10], and used to study receptor complexes. However, these fluorescent tracers are relatively nonselective within the AR family.

Receptor-selective fluorescent ligands are used increasingly as tools for the study of receptor physiology and pathophysiology at the cellular and even the subcellular level [7]. Furthermore, they are being investigated as screening tools in drug discovery [6]. In both cases, techniques employing receptor-selective fluorescent ligands have proved to be complementary to, and in several cases even superior to, the traditional radioligand-based techniques. Increasing costs and public concerns associated with radioactive isotope handling and disposal are also making the use of fluorescent ligands more attractive in research and diagnostics. With the increasing importance of GPCRs in pharmacology and the search for newer, more potent and/or selective receptor ligands and drugs, there is an accompanying need for the design and development of novel highly potent and selective fluorescent ligands. Such ligands will likely aid in the investigation of the physiological and pathophysiological functions of GPCRs and also in the development of drugs acting specifically at these receptors.

We recently introduced a versatile ligand for fluorescence polarization (FP) studies of the A_{2A} AR, the fluorophore-conjugated tricyclic derivative MRS5346 [11]. This ligand was selective for that AR subtype and was not suitable for measurements of the A_3 AR due to low affinity. In this study, we have explored the SAR of chain-elongated derivatives of known AR antagonists, including the pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-ylamine class (designated here as pyrazolo-triazolo-pyrimidine, PTP), already reported to display A_3 AR selectivity [12]. We also prepared a fluorescent derivative of the closely related triazolo[1,5-c]quinazolin-5-ylamine class (designated here as triazolo-quinazoline, TQ), of which the potent, but nonselective AR antagonist (*N*-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-yl)amine (CGS15943, **7**, Figure 1) is a member [13,14]. We discovered that an Alexa Fluor-488 derivative (MRS5449, **9**) of a TQ antagonist, in which the fluorophore was coupled to an extended chain using a click reaction [15], displayed high affinity at the human (h) A_3 AR and was suitable as a molecular probe in flow cytometry (FCM) studies.

FCM is a sensitive and reliable method for examining and counting fluorophore-tagged whole cells. It is routinely used in clinical practice (e.g. diagnosis of health disorders such as leukemia [16]) and has many applications in drug research (e.g. nucleic acid analysis [17]), but its application to quantitative receptor binding studies has been limited [18]. However, for quantitative molar measurements, the mean fluorescence intensity (MFI) of fluorescent small molecules bound to a cell can be measured as well, which is a relative value directly proportional to the number of receptor-bound molecules can be converted to a value of molecules of equivalent soluble fluorochrome (MESF) using quantitative fluorescence calibration (QFC) [19,20]. Therefore, comparable, instrument-independent fluorescence data

can be calculated using a standard to provide pharmacological parameters of the binding of a fluorescent ligand to intact cells expressing the hA₃AR.

2. Materials and Methods

2.1. Materials

Alexa Fluor-488 5-carboxamido-(6-azidohexanyl)bis(triethylammonium salt) was purchased from Invitrogen (Carlsbad, CA). [³H]R-*N*⁶-(2-phenylisopropyl)adenosine ([³H]R-PIA, 42.6 Ci/mmol) was obtained from Moravsek Biochemicals (Brea, CA). [¹²⁵I]4-Amino-3-iodobenzyl-5'-N-methylcarboxamidoadenosine ([¹²⁵I]I-AB-MECA, 2200 Ci/mmol), and [³H]-2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine ([³H]CGS21680, 40.5 Ci/mmol) were purchased from Perkin Elmer (Waltham, MA). DMEM/F12 medium and 1 M Tris-HCl (pH 7.5) were purchased from Mediatech, Inc. (Herndon, VA). Adenosine deaminase, Cl-IB-MECA and NECA were from Sigma (St. Louis, MO). CGS15943, CGS21680, CPA, DPCPX, IB-MECA, MRS1220, PSB10, XAC and ZM241385 were from Tocris (Ellisville, MO). Quantum Alexa Fluor-488 MESF beads were purchased from Bangs Laboratories, Inc. (Fishers, IN). All other reagents were from Sigma-Aldrich (St. Louis, MO), unless noted, and were of analytical grade and used without further purification.

2.2. Chemical synthesis

The synthesis derivatives **1** – **6**, including conjugation with fluorescein isothiocyanate, isomer 1 (FITC), will be described in detail elsewhere.

General methods—Reactions were conducted under an atmosphere of argon whenever anhydrous solvents were used. All reactions were monitored by thin-layer chromatography (TLC) using silica gel coated plates with a fluorescence indicator (Sigma-Aldrich, St. Louis, MO), which was visualized under UV light. Silica gel column chromatography was performed with silica gel (SiO₂, 200–400 mesh, 60 Å, Sigma-Aldrich, St. Louis, MO) using moderate air pressure. Evaporation of solvents was carried out under reduced pressure at a temperature below 40 °C. After column chromatography, appropriate fractions were pooled, evaporated and dried at high vacuum for at least 12 h to give the desired products in high purity. ¹H NMR and analytical HPLC ascertained sample purity. No corrections in yield were made for solvent of crystallization. ¹H NMR spectra were recorded at 400 MHz. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane or deuterated solvent as the internal standard (δH: CDCl₃ 7.26 ppm). High resolution mass spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine. Observed mass accuracies are those expected on the basis of known performance of the instrument as well as the trends in masses of standard compounds observed at intervals during the series of measurements. Reported masses are observed masses uncorrected for this time-dependent drift in mass accuracy.

2.2.1. *N*-(9-Chloro-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-yl)hex-5-ynamide (8**):** Compound **7** (11.2 mg, 39.2 μmol) was co-evaporated with anhydrous toluene (2 × 5 ml) and dissolved in anhydrous CH₂Cl₂: *N,N'*-dimethylformamide (1 ml, 1:1, v/v). 1-Ethyl-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC.HCl, 45 mg, 235 μmol), 5-hexynoic acid (26 μl, 235 μmol), 4-dimethylaminopyridine (2 mg, 15.6 μmol) and triethylamine (82 μl, 588 μmol) were added. The reaction mixture was stirred for 22 h whereupon it was diluted with CH₂Cl₂ (30 ml) and sequentially washed with brine (2 × 10 ml) and distilled water (2 × 10 ml). The organic phase was evaporated to dryness and the resulting residue purified by silica gel column chromatography (0–25% acetone in hexane,

v/v) to afford compound **8** as a white solid (12.1 mg, 81%). $R_f = 0.5$ (30% acetone in hexane, v/v); MALDI-HRMS m/z 380.0919 ($[M+H]^+$, $C_{19}H_{14}N_5O_2Cl \cdot H$ Calcd 380.0914); Selected signals: 1H NMR ($CDCl_3$) δ 9.03 (s, 1H), 8.51 (d, $J = 2.3$ Hz, 1H), 7.91 (d, $J = 8.8$ Hz, 1H), 7.76 (dd, $J = 8.8$ & 2.3 Hz, 1H), 7.70 (s, 1H), 7.33 (d, $J = 3.5$ Hz, 1H), 6.64–6.67 (m, 1H), 3.25 (t, $J = 7.3$ Hz, 2H), 2.42–2.48 (m, 2H), 2.03–2.13 (m, 3H). Analytical purity of compound **8** was determined using a Hewlett–Packard 1100 HPLC equipped with a Zorbax SB-Aq 5 μ m analytical column (50 $\times$ 4.6 mm; Agilent Technologies Inc, Palo Alto, CA). Mobile phase: linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogen phosphate)- CH_3CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 ml/min. Peaks were detected by UV absorption with a diode array detector at 254, 275, and 280 nm. Purity >99% by HPLC (retention time: 10.43 min).

2.2.2. 2-(6-Amino-3-iminio-4,5-disulfonato-3H-xanthen-9-yl)-5-((6-(4-(4-((9-chloro-2-(furan-2-yl)-[1,2,4]triazolo[1,5-c]quinazolin-5-yl)amino)-4-oxobutyl)-1H-1,2,3-triazol-1-yl)hexyl)carbamoyl)benzoate (9, MRS5449): A solution of Alexa Fluor-488 5-carboxamido-(6-azidohexanyl)bis(triethylammonium salt) (0.5 mg, 0.58 μ mol, Invitrogen-Life Technologies, Grand Island, NY) in H_2O (200 μ l) was added to a solution of alkyne **8** (0.44 mg, 1.1 μ mol) in N,N' -dimethylformamide (100 μ l). A freshly prepared solution of aqueous sodium ascorbate (0.1 M, 8 μ l, 5.86 μ mol) was added to the reaction mixture followed by addition of aqueous cupric sulfate pentahydrate solution (0.1 M, 2.94 μ l, 0.29 μ mol). The resulting mixture was stirred overnight at room temperature; 10 mM aqueous solution of triethylammonium acetate buffer (2 ml) was added with constant mixing. The resulting mixture was lyophilized and purified by HPLC with a Luna 5 μ RP-C18 semipreparative column (250 $\times$ 10.0 mm; Phenomenex, Torrance, CA) under the following conditions: flow rate of 2 ml/min; H_2O - CH_3CN from 100:0 (v/v) to 0:100 (v/v) in 30 min and isolated at 20.07 min to get Alexa Fluor-488 conjugate **9** (0.16 mg, 27%). ESI-HRMS m/z 1036.1957 $[M + Na]^+$, $C_{46}H_{38}N_{11}O_{12}S_2Cl \cdot H^+$: Calcd. 1036.1910. Analytical purity >99% by HPLC (retention time: 10.13 min).

2.3. Cell cultures and membrane preparation—Chinese hamster ovary (CHO) cells without heterologous AR expression were grown in DMEM/F12 (1:1) medium with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin. CHO cells expressing the A_1 or A_3 AR and HEK293 cells expressing the A_{2A} AR were grown in the same medium but in the presence of 500 μ g/ml hygromycin B and 800 μ g/ml of G418, respectively. Cells, used for FCM analysis, were grown in 6-well plates (approximately 400,000 cells/well) and incubated at 37 $^\circ$ C for 24 h in the presence of 5% CO_2 . When the confluency of the cells reached 80% (approximately 10^6 cells/well), medium was replaced with fresh medium and MRS5449 was added in the presence or absence of competitive antagonists, and cells were processed for FCM.

For cell cultures used in radioligand binding assays, after harvest and homogenization, the cells were centrifuged at $200 \times g$ for 10 min, and 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 $20,000 \times g$ for 20 min at 4 $^\circ$ C. The resultant membrane pellets were resuspended in buffer in the presence of 3 units/ml adenosine deaminase, and the suspension was stored at -80° C until the binding experiments. The protein concentration was measured using the Bradford assay [21].

2.4. Radioligand membrane binding assays—Radioligand binding assays at hA_1 , hA_{2A} , and hA_3 ARs were performed according to the procedures described previously [22] using the radiolabeled agonists [3H]R-PIA from Moravak Biochemicals (Brea, CA) or [3H]CGS21680 and [^{125}I]I-AB-MECA from PerkinElmer (Waltham, MA) for the hA_1 , hA_{2A} AR, and hA_3 AR assays, respectively.

2.5. Cyclic AMP accumulation assay—CHO cells stably expressing the hA₃AR were seeded in 24-well plates and incubated at 37°C overnight. The medium was removed the following day and replaced with DMEM containing 50 mM HEPES, 10 μM rolipram, 3 units/ml adenosine deaminase (Worthington Biochemical Corp., Lakewood, NJ), and increasing concentrations of agonists and incubated for 30 min. Forskolin (10 μM) was added and incubated for an additional 15 min. The medium was removed, and the cells were lysed with 200 μL of 0.1 M HCl. 100 μL of the HCl solution was used in the Sigma Direct cAMP Enzyme Immunoassay following the instructions provided with the kit. The OD values were measured with a SpectraMax M5 Microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm.

2.6. Fluorescence microscopy experiments—CHO cells stably expressing the hA₃AR were grown on sterile coverslips in 6-well plates, and experiments were performed when the cells reach 70% confluency after refreshing the medium. The cells were incubated with 50 nM MRS5449 for different time intervals from 15 min to 3 h at 37 °C in an atmosphere containing 5% CO₂. At the end of each time interval, the medium was removed, and cells were washed three times with ice-cold PBS (Crystalgen, Commack, NY). The coverslips containing the cells were placed on sterile slides, and the cells were observed under a Zeiss AxioCam MRm fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

2.7. Fluorescent ligand binding in intact CHO cells expressing the A₃AR—In preliminary binding experiments of compounds **1–6** and MRS5449 were incubated with CHO cells expressing the hA₃AR for 1 h at 37°C in an atmosphere containing 5% CO₂. The concentrations used were incubated with 1.5 μM **1**, 5 μM **2**, 1.5 μM **3**, 10 μM **4**, 20 μM **5**, 15 μM **6**, or 10 nM MRS5449. For further binding experiments with MRS5449, control CHO cells (CHO cells without heterologous receptor expression) and CHO cells expressing hA₃AR were incubated with 10 nM MRS5449 for 3 h.

In saturation binding experiments, CHO cells expressing the hA₃AR were incubated with concentrations of MRS5449 ranging from 2.5 nM to 250 nM for 1 h. To study binding kinetics of MRS5449, we incubated CHO cells expressing the hA₃AR with 10 nM MRS5449 for different time intervals from 5 min to 3 h. The association and dissociation rate was measured as follows: 10 nM MRS5449 was added to CHO cells expressing the A₃AR and measured total binding at different time intervals. After 90 min, we initiated dissociation by adding 10 μM of non-fluorescent A₃AR antagonist MRS1220 and then measured the dissociation at different time intervals.

To determine binding affinities of known AR ligands using MRS5449 as a tracer, hA₃AR-expressing CHO cells were incubated with different concentrations of ligands in the presence of 5 nM MRS5449 for 90 min. Total binding was measured in the absence of the competitor. Nonspecific binding was determined in the presence of 10 μM MRS1220.

At the end of each time interval, the medium was removed and cells were washed three times with ice-cold DPBS. After washing, 1 ml 0.2 % EDTA solution was added to each well, and cells were incubated at 37 °C for 7 min. Following cell detachment, 1 ml medium was added to each well to neutralize the EDTA. The cell suspensions were transferred to polystyrene round-bottom BD Falcon tubes (BD, Franklin Lakes, NJ) and centrifuged for 5 min at 23 °C and 400×g. After centrifugation, the supernatant was discarded, and cells were washed with 3 ml PBS and centrifuged again at 23 °C and 400×g for 5 min. After discarding the supernatant, cells were suspended in 0.5 ml PBS and analyzed by FCM.

2.8. FCM calibration—A measurable intrinsic parameter of a fluorescent ligand or particle is its fluorescence yield, which can be expressed as molecular equivalent values of

free fluorophore in solution (MESF). Standard particles suspensions with assigned MESF values, e.g. Quantum Alexa Fluor-488 MESF beads (Bangs Laboratories, Inc., Fishers, IN), establish an instrument-independent scale that accurately depicts the molar quantity of fluorophores on labeled particles.

The mean fluorescence intensity (MFI) values of the standard particles were recorded under optimized instrument setting and converted into MESF values using the QuickCal program v. 2.3 (Bangs Laboratories, Inc., Fishers, IN). MFIs of receptor-bound fluorescent small molecules in this study were measured under exactly the same conditions and the assigned MESF values were extrapolated from the calibration curve. Further details of MESF calibration can be found in Supporting Information.

2.9. FCM analysis—The intensity of fluorescence emission of each sample was measured by using FCM. Cell suspensions were vortexed briefly before analysis on a Becton and Dickinson FACSCalibur flow cytometer (BD, Franklin Lakes, NJ) with excitation at 488 nm. Samples were maintained in the dark during the analysis to avoid photobleaching. MFIs were obtained in the FL-1 channel in log mode. Ten thousand events were analyzed per sample. Data were collected using Cell Quest Pro software (BD, Franklin Lakes, NJ) and analyzed by Cyflogic v. 1.2.1 software (CyFlo, Ltd., Turku, Finland).

2.10. Data analysis—Data analysis was performed with the Prism 5 (GraphPad, San Diego CA) software. The mean autofluorescence of CHO cells was measured in the absence of the fluorescent ligand. The mean fluorescence in the presence of fluorescent ligand was corrected by subtracting this value [23]. Further details of data analysis can be found in Supporting Information.

2.11. Molecular modeling—We used our recently built homology model of the hA₃AR [24], based on the crystallographic structure of an antagonist-bound hA_{2A}AR [25], to identify the hypothetical binding modes of the new fluorescent derivatives.

In particular, a two-step approach has been used due to the large conformational flexibility of these ligands. In the first step, molecular docking simulations were performed using both PTP and TQ scaffolds only derivatized through the exocyclic amine with a C4-alkyl chain (i.e. *n*-butyl for PTP and *n*-butanoyl for TQ) that does not include the corresponding fluorophores. Molecular docking studies have been carried out at the hA₃AR binding site using the docking software Gold (version 5.1) [26]. The electrostatic and hydrophobic contributions to the interaction energy of each receptor residue involved in the binding with these fragments were calculated using the software MOE (version 2010.10) [27].

Starting from top-scoring poses of both PTP and TQ simplified analogs, the complete ligands linked to the corresponding fluorophores were constructed by elongating the C4-alkyl spacer in the direction of the extracellular environment. Finally, an exhaustive conformational analysis of the fluorescent ligands along with extracellular loop 2 (EL2) of the protein (starting from Asn150 to Gln167) was performed, using the LowMode MD search method implemented in MOE [28]. Detailed information regarding molecular modeling methodologies is provided in Supporting Information.

3. RESULTS

3.1. Synthesis and characterization of fluorescent AR antagonists

3.1.1. Synthetic approaches—Known heterocyclic antagonists of the A₃AR of high affinity were derivatized through the exocyclic amine with chemically functionalized chains and conjugated to fluorophores of the fluorone class. PTP derivatives of varying chain

length **1** – **6** were prepared by coupling FITC to various chain-extended alkyl amino derivatives, in which the anchoring point was the exocyclic amine common to many structural classes of AR antagonists. FITC was used previously in a fluorescent agonist conjugate designed for binding to the A_{2A}AR [29]. Derivatives **2** – **4** contained homologous *n*-alkyl spacer chains, and **5** and **6** contained multiple ether linkages. The shortest member of the series, **1**, is a hydrazino derivative with no alkyl spacer chain, and the longest member, **6**, contains a spacer chain of 13 atoms.

As an alternative to the known, generally A₃AR-selective PTP scaffold, we explored the use of the TQ scaffold. It is known that acylations at the exocyclic amine of a nonselective triazolo[1,5-*c*]quinazolin-5-yl)amine AR antagonist, CGS15943, provide varying degrees of A₃AR selectivity [14]. We also used an alternate coupling approach of click chemistry to label the scaffold with an analogue of fluorescein, i.e. the fluorophore Alexa Fluor-488, as was used in our previous FP study of the A_{2A}AR [11]. Its spectral properties are nearly identical to those of fluorescein, over which it has many advantages for FCM experiments, e.g. photostability, brightness, relative pH-insensitivity and a relatively high Stokes shift.

Click chemistry is a facile means of combining two chemical moieties, even if divergent in molecular weight or chemical properties [15]. We have adapted the most commonly used form of click chemistry here for the synthesis of TQ derivative MRS5449, i.e. copper(I) catalyzed 3+2 cycloaddition of an azide and a terminal alkyne group [15]. This reaction forms a triazole linkage, which is expected to be more chemically stable than the thiourea group present in the PTP derivatives **1** – **6** and also makes it possible to interact with potential aromatic binding residues in the outer region of the receptor binding pocket [25].

In order to introduce the handle with a spacer arm for attachment of the Alexa Fluor-488 fluorophore, commercially available compound **7** (CGS15943) was initially condensed with 5-hexynoic acid using EDC to obtain the terminal alkyne derivative **8** in 80% yield. The fluorophore was attached to the terminal alkyne of compound **8** using click reaction conditions to obtain target conjugate MRS5449 in a yield of 26%.

3.1.2. Pharmacological and spectral characterization of newly synthesized AR antagonist ligands—The binding affinity of these derivatives at three subtypes of hARs was determined using standard radioligand binding methods, and the K_i values are shown in Table 1. The binding affinities of the conjugates **1** – **6** show that roughly μM affinity was not exceeded at three AR subtypes. The most potent hA₃AR ligands in the group of PTP derivatives were **1** and **3** with K_i values of 780 and 720 nM, respectively, in a binding assay using [¹²⁵I]I-AB-MECA as a radioligand. The hA₃AR binding of TQ derivative MRS5449 displayed a K_i value of 6.4 nM (Figure 2A). The affinity of MRS5449 at two other ARs was lower; at hA₁ and hA_{2A}ARs, the K_i values in inhibition of radioligand binding were 87 nM and 73 nM, respectively. Thus, the selectivity for the A₃AR was over 10-fold. The effect of chain extension at the exocyclic amine of **7**, as found in **8** or when terminated in a click-conjugated fluorophore in MRS5449, was to greatly reduce hA₁ and hA_{2A}AR affinity while maintaining nearly identical A₃AR affinity. Moreover the conjugation of the fluorophore increased the hA₃AR affinity.

In a functional assay of cyclic AMP accumulation in CHO cells expressing the A₃AR, MRS5449 concentration-dependently antagonized the effects of A₃AR agonist Cl-IB-MECA (Figure 2B) corresponding to a K_B value of 4.8 nM (Figure 2C). MRS5449 displayed excitation and emission peaks at 494 and 523 nm, respectively. Fluorescent characterization of the conjugates **1** – **6** is available in Supporting Information.

In preliminary FCM experiments, the binding affinity of compounds **1–6** and MRS5449 at the hA₃AR expressed on CHO cells was measured by recording MFI values of the cell-fluorescence in the presence of small molecule conjugates and converting them into MESF values. However, conjugates **1 – 6** resulted in very low fluorescence emission, and only MRS5449 produced a sufficiently high MFI for further investigations in fluorescent experiments, even at 250 to 1000-fold lower concentration than compounds **1 – 6** (Figure 3). Compared with the binding to the A₃AR (expressed as 100%), the respective binding of MRS5449 to CHO cells expressing the A₁AR and HEK cells expressing the A_{2A}AR was only 0.3% and 0.5%.

According to its fluorescent properties, binding affinity and selectivity at the hA₃AR, MRS5449 seemed to be a preferred ligand for further examination as a fluorescent tracer in various assay modes.

3.2. Fluorescence microscopy experiments

Fluorescence micrographs showing the binding of 50 nM MRS5449 to the A₃AR expressed in CHO cells at different time points are shown in Figure 4. The fluorescence was highly associated with the plasma membrane, and internalized MRS5449 did not appear to be the source of most of the fluorescence. The corresponding control experiments, performed either in the absence of MRS5449 or in its presence except with preincubation with hA₃AR antagonist MRS1220 (10 μM), did not indicate any appreciable bound fluorescence. The intensity of fluorescence gradually increased over an incubation period of 15 min – 180 min.

3.3. FCM assays with MRS5449

3.3.1. FCM calibration—We analyzed the fluorescence intensities of AlexaFluor-488 labeled standard beads with assigned MESF values at five different fluorescence intensity levels. This was used to establish a calibration curve to relate relative fluorescence signals to absolute fluorescence that was translated to molar concentration of the fluorophore (Figure S10, Supporting Information). By converting MFI values into MESF values based on this calibration curve, molar quantitation was accomplished.

3.3.2. Fluorescent ligand binding experiments—In a saturation binding experiment, we varied the concentration of fluorescent ligand MRS5449 from 2.5 nM to 250 nM and measured MFI of the receptor bound ligand. A saturation binding curve was obtained using MESF values, since FCM is insensitive to unbound soluble ligand (Figure 5A). As a negative control we used CHO cells expressing hA₃AR in the absence of fluorescence ligand (autofluorescence). Nonspecific binding of 10 nM MRS5449 measured in the presence of 10 μM antagonist MRS1220 or 10 μM agonist Cl-IB-MECA or in control CHO cells (without heterologous receptor expression) was less than 1% of total binding. A FCM histogram of the total binding and nonspecific binding to hA₃AR expressing CHO cells using MRS5449 at the suitable tracer concentration of 10 nM is shown in Figure 5B. Total binding was significantly higher than the nonspecific binding in antagonist-treated cells, which was similar to the autofluorescence of the cells. The peak of the total binding located within the optimal window for analysis and was well separated from the levels of nonspecific binding and the autofluorescence. From the saturation curve, the equilibrium binding constant (K_d) was determined to be 5.15 ± 1.11 nM, which is similar to a K_i value of 6.4 nM obtained from radioligand binding experiments.

We next determined using an FCM assay the association kinetics of the fluorescent ligand MRS5449. Figure 5C shows the time-dependent binding of 10 nM MRS5449 to the hA₃AR. The $t_{1/2}$ for association was found to be 53 min.

Dissociation was determined by adding 10 μ M MRS1220 after equilibrium was reached (Figure 5D). The K_d value determined through association and dissociation kinetic experiments is 6.65 ± 0.55 nM, which correlated well with the equilibrium binding constant determined in a saturation binding experiment and also closely matched the K_i value in radioligand binding assays.

3.3.3. Screening of known AR ligands using MRS5449 as a tracer—Competition for fluorescent binding with known AR ligands was performed using FCM. To determine the K_i values of known ligands, we used MRS5449 as a tracer, at a concentration of 5 nM (close to its K_d value). Competitive binding results with known agonists and chemically diverse antagonists are shown in Figure 6A and Table 2. The observed pharmacology of antagonists in this whole cell FCM assay corresponded to that previously observed for the hA₃AR. However, K_i values of known AR agonists in inhibition of MRS5449 binding were roughly 5–20 fold weaker than in agonist radioligand binding experiments in membranes. A further detailed examination of the displacement curve of CI-IB-MECA for the antagonist fluorescent ligand MRS5449 suggests that multiple agonist binding states of the hA₃AR can be demonstrated (Figure 6B). Analysis the binding curve with a two-site binding model shows that CI-IB-MECA binds to a high-affinity binding component corresponding to a K_i value 0.1 nM, i.e. closer to the K_i value determined using an agonist radioligand [33]. A low affinity binding component for CI-IB-MECA was determined to correspond to a K_i value of 136 nM (Figure 6B).

3.4. Molecular modeling studies

We performed molecular modeling studies with the aim to identify the binding modes of the newly synthesized fluorescent derivatives at the hA₃AR and to understand the structural basis for the affinity enhancement of MRS5449. For these studies we used a recently built homology model of the hA₃AR [24], based on the crystallographic structure of an antagonist-bound hA_{2A}AR [25].

Due to the conformational complexity of the ligands, we applied a two-step approach consisting of molecular docking of the ligand scaffolds followed by conformational searching of fluorophore-chain fragments inside the hA₃AR binding site. In the first step, molecular docking simulations were performed using both PTP and TQ scaffolds derivatized only through their exocyclic amines with a C4-alkyl chain and without including the corresponding fluorophores. Binding poses at the hA₃AR of PTP and TQ simplified analogs, along with their *per residue* electrostatic and hydrophobic contributions graphs are reported in Supporting Information.

Interestingly, analysis of top-scoring docking poses revealed that PTP and TQ scaffolds possessed a similar binding mode in the TM region of the hA₃AR. Ligands recognition occurred in the upper region of the TM bundle, and the tricyclic nuclei were surrounded by TMs 3, 5, 6, and 7 with the furyl rings directed toward TM 2. Moreover, PTP and TQ scaffolds showed similar crucial interactions with residues of the binding site, such as two hydrogen bonds with Asn250 (6.55) and an aromatic π - π stacking interaction with Phe168 (EL2) (see Supporting Information). These observed similar interaction patterns were confirmed by the analysis of calculated electrostatic and hydrophobic contributions to the interaction energy of each receptor residue involved in the binding with these fragments. Comparison of these contributions for the two binding poses pointed out high similarities with only slight differences. In fact, favorable electrostatic contributions to the binding energy were stronger for the PTP fragment binding pose, while favorable hydrophobic contributions to the binding energy were stronger for the TQ fragment binding pose (see Supporting Information).

Starting from the top-scoring pose of each of the PTP and TQ simplified analogs, the corresponding complete ligands linked to the corresponding fluorophores were constructed by elongating the C4-alkyl spacer in the direction of the extracellular environment. Afterwards, an exhaustive conformational analysis of the fluorescent ligands along with EL2 of the protein (starting from Asn150 to Gln167) was performed, using the LowMode MD search method implemented in MOE [28].

Structures of the other ELs of the hA₃AR (EL1, EL3) were kept fixed, while conformational search was performed for EL2, because it consists of a long sequence, and its conformation is consequently highly uncertain in the model.

Therefore, this procedure allowed us to explore the conformations of the fluorophore-chain fragments that have the most favorable interactions with EL2 and with the outer part of the binding site. Detailed binding conformations at the hA₃AR of MRS5449 and compound **3** obtained after LowMode MD search are reported in Supporting Information. However, a schematic representation of these two ligand-receptor complexes, embedded in a solvated lipid bilayer simulating the cell membrane, is displayed in Figure 7. Analysis of the obtained conformations showed that compound **3** was able to form only a few interactions with the residues at the entrance of the hA₃AR binding site, but was unable to interact with residues of EL2. This seemed to be due to the insufficient length of the chain departing from the exocyclic amine that precluded the fluorophore reaching and interacting with the tip of EL2.

On the contrary, the Alexa Fluor-488 fluorophore of MRS5449 strongly interacted, mainly through its amino and sulfonate groups, with several residues of EL2, including His158, Arg159, Asn160, Ser165 and Gln167. It is to be noted that, given the flexible nature of EL2, the highly uncertain conformation of the EL region in the model and the flexible nature of the ligand linker, it is difficult to identify a detailed and robust binding mode for these fluorescent ligands. However, the performed conformational search highlighted important differences in the interactions of compound **3** and MRS5449 with the outer region of the hA₃AR binding site.

Therefore, considering the results of this computational analysis, we can speculate that both the increased chain length and the extra functionality on the fluorophore of MRS5449 seemed to establish a more favorable interaction of this conjugate with the hA₃AR and its higher affinity in comparison to PTP derivatives **1–6**. As shown in Figure 7, the Alexa Fluor-488 fluorophore of MRS5449, thanks to appropriate length of its linker, was located in the outer loop area of the hA₃AR, entirely outside of the TM region. Thus, this ligand displayed excellent structural features for its application as a fluorescent probe in pharmacological experiments to characterize the hA₃AR.

4. DISCUSSION

We have shown that the bivalent ligand MRS5449, containing a high affinity antagonist moiety coupled to a widely used fluorophore, can serve as a receptor subtype-selective molecular probe for FCM experiments on intact cells expressing the hA₃AR. This now adds to a growing list of fluorescent probes for the ARs [9–11,29]. Although the selectivity in comparison to the hA₁AR and hA_{2A}AR was only moderate, it seemed sufficient to provide a selective FCM signal corresponding to the hA₃AR. We have not evaluated this molecular probe at ARs in other species, but it is expected that the affinity at the rat and mouse A₃AR will be considerably lower based on known SAR of the TQ series at ARs [12].

Thus, a fluorescence assay using FCM can be a useful tool in hA₃AR binding studies. Compared to radioligand binding assays using isolated cell membranes and at room temperature, this FCM approach using a 37°C incubation of intact cells is a novel technique

to study ARs closer to their native environment. Using MRS5449 as a tracer, the agonist-induced changes of ARs in intact cells (such as desensitization, internalization or downregulation) may be followed during the binding process. Moreover, the low nonspecific binding of MRS5449 and the high sensitivity of the FCM enables this approach to be suitable for the physiological and pathophysiological characterization of hA₃AR and the pharmacological characterization of its ligands.

A disadvantage of this FCM method is that it is not suitable for high throughput assay and is more technically demanding than conventional radioligand binding technique [32]. However, in studies requiring the characterization of hA₃AR ligands in their native environment, the FCM method is a preferable choice.

It should be noted that in the present study, both the association and dissociation of MRS5449 were slower compared with those from radioligand binding to cell membrane preparations. It is not clear if this reflects a property of this fluorescent ligand or is a characteristic of A₃AR binding in intact cells. Slow rate constants have been noted for the binding of other fluorescent GPCR ligands.

Unfortunately, none of the affinities of the PTP derivatives reached the nM range at any of the ARs. We have not directly compared the PTP scaffold with a TQ scaffold in the otherwise identical conjugate series. Therefore, we cannot establish the precise structural basis for the selectivity of MRS5449 in comparison to PTP derivatives **1** – **6**. However, as shown by molecular modeling, both the chain length and the extra functionality on the fluorophore (i.e. amino and sulfonate groups) of MRS5449 are likely crucial features for the binding of this ligand at the hA₃AR. According to the present study, the Alexa Fluor-488 fluorophore of MRS5449 is predicted to strongly interact with residues located at the tip of EL2 of the hA₃AR, such as His158, Arg159 and Asn160.

The hA₃AR is overexpressed in various forms of cancer and inflammation [1,2]. The expression level of the A₃AR in peripheral blood mononuclear cells is also indicative of the disease state [34]. Therefore, a FCM approach to the characterization of the surface expression of the A₃AR could be useful as a diagnostic indicator in a clinical setting.

In summary, we have developed a potent and selective fluorescent ligand, MRS5449, by conjugating the AR antagonist CGS15943 and the Alexa Fluor-488. MRS5449 has been used successfully in quantifying the hA₃AR, studying receptor kinetic properties, and ligand screening in intact cells using FCM. A similar approach can be applied to develop fluorescent ligands for other AR subtypes and other GPCRs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AF488

Alexa Fluor-488

CHO	Chinese hamster ovary
CGS15943	<i>N</i> -[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5- <i>c</i>]quinazolin-5-amine
CGS21680	2-[<i>p</i> -(2-carboxyethyl)phenylethylamino]-5'- <i>N</i> -ethylcarboxamido-adenosine
CI-IB-MECA	1-[2-chloro-6-[[3-(iodophenyl)methyl]amino]-9 <i>H</i> -purin-9-yl]-1-deoxy- <i>N</i> -methyl-β-D-ribofuranuronamide
CPA	(2 <i>R</i> ,3 <i>R</i> ,4 <i>S</i> ,5 <i>R</i>)-2-[6-(cyclopentylamino)purin-9-yl]-5(hydroxymethyl)oxolane-3,4-diol
DMAP	4-dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
PBS	phosphate buffered saline
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
EDC	1-ethyl-[3-dimethylaminopropyl]carbodiimide
EDTA	ethylenediaminetetraacetic acid
EL	extracellular loop
FBS	fetal bovine serum
FCM	flow cytometry
FITC	fluorescein isothiocyanate
GPCR	G protein-coupled receptor
HEK	human embryonic kidney
[¹²⁵I]I-AB-MECA	[¹²⁵ I]4-amino-3-iodobenzyl-5'- <i>N</i> -methylcarboxamidoadenosine
IB-MECA	1-Deoxy-1-[6-[[3-(iodophenyl)methyl]amino]-9 <i>H</i> -purin-9-yl]- <i>N</i> -methyl-β-D-ribofuranuronamide
MD	molecular dynamics
MESF	molecules of equivalent soluble fluorochrome
MOE	Molecular Operating Environment
MFI	mean fluorescent intensity
MRS1220	<i>N</i> -[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5- <i>c</i>]quinazolin-5-yl]benzene acetamide
MRS5346	5-((2-(2-(4-(3-(5-amino-2-(furan-2-yl)-7 <i>H</i> -pyrazolo[4,3- <i>e</i>][1,2,4]triazolo[1,5- <i>c</i>]pyrimidin-7-yl)propyl)phenoxy)acetamido)-ethyl)carbamoyl)-2-(6-amino-3-iminio-4,5-disulfonato-3 <i>H</i> -xanthen-9-yl)benzoate
MRS5449	2-(6-amino-3-iminio-4,5-disulfonato-3 <i>H</i> -xanthen-9-yl)-5-((6-(4-(4-(9-chloro-2-(furan-2-yl)-[1,2,4]triazolo[1,5- <i>c</i>]quinazolin-5-

	yl)amino)-4-oxobutyl)-1 <i>H</i> -1,2,3-triazol-1-yl)hexyl)carbamoyl)benzoate
NECA	5'- <i>N</i> -ethylcarboxamidoadenosine
PSB10	8-ethyl-1,4,7,8-tetrahydro-4-methyl-2-(2,3,5-trichlorophenyl)-5 <i>H</i> -imidazo[2,1- <i>i</i>]purin-5-one monohydrochloride
TM	transmembrane helix
XAC	xanthine amine congener, <i>N</i> -(2-aminoethyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1 <i>H</i> -purin-8-yl)phenoxy]-acetamide hydrochloride
ZM241385	4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3- <i>a</i>][1,3,5]triazin-5-ylamino]ethyl)phenol

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Figure 1.

The chemical structures of fluorescent (A) PTP and (B) TQ derivatives. The route to synthesis of TQ derivative MRS5449, **9** is shown. Reagents and Conditions: a) 5-hexynoic acid, EDC.HCl, DMAP, Et₃N, an CH₂Cl₂:DMF (1:1, v/v), 81%; b) Alexa Fluor-488 5-carboxamido-(6-azidohexanyl) bis(triethylammonium salt), aq. sodium ascorbate, aq. cupric sulfate pentahydrate solution, H₂O, DMF, 26%.

Figure 2.

A) Displacement by MRS5449 of radioligand binding to membrane preparations from CHO and HEK293 cells stably expressing hARs. Results are from one experiment representative of three independent experiments performed in duplicate. The K_i values from three experiments are listed in Table 1. B) Rightward-shift by MRS5449 of the concentration-response curve of the A_3AR agonist Cl-IB-MECA in inhibition of forskolin-stimulated cyclic AMP production in CHO cells expressing the A_3AR . C) Schild analysis of the effect of the fluorescent antagonist MRS5449 on inhibition of cyclic AMP accumulation induced by the A_3AR agonist Cl-IB-MECA in CHO cells expressing the A_3AR . The K_B value was calculated to be 4.8 nM.

Figure 3.

Fluorescence ligand binding experiments with compound **1** – **6** and MRS5449 with FCM. hA_3AR -expressing CHO cells were incubated with fluorescent small molecule conjugates were used in a concentration approximately two times higher than its K_i values, and mean fluorescence intensities (MFI) of the cell-small molecule conjugates were measured with FACSCalibur flow cytometer. MFI values were converted into MESF values using QuickCal program v. 2.3 after correcting for autofluorescence. Percentages above each column show the brightness of each compound using MRS5449 set at 100%. Results are expressed as mean $\pm$ S.E. (n=3).

Figure 4.

Fluorescence micrograph of CHO cells expressing the hA₃AR following incubation with 50 nM MRS5449 for the time as indicated: A) 15 min; B) 60 min; C) 120 min; D) 180 min. hA₃AR-expressing CHO cells preincubated with 10 μM MRS1220 (E) and hA₃AR-expressing CHO cells in the absence of any fluorescence ligand (F) were used as controls, but significant cell fluorescence was not observed.

Figure 5.

Fluorescence binding experiments with MRS5449 using FCM in CHO cells expressing hA₃AR. MFI of the cell-small molecule conjugates were measured with FACSCalibur flow cytometer. MFI values were converted into MESF values using QuickCal program v. 2.3 after correcting for autofluorescence. A) Saturation binding of MRS5449. Nonspecific binding was measured in the presence of 10 μ M selective hA₃AR antagonist MRS1220. The K_d value was determined to be 5.15 ± 1.11 nM. Results are expressed as mean $\pm$ S.E. (n=3). B) Histogram of the total binding (blue), nonspecific binding (red) and autofluorescence (green) of 10 nM MRS5449 to CHO cells expressing hA₃AR after 90 min incubation. The histogram represents data of one of the three independent experiments. C) Association binding kinetics of MRS5449 to CHO cells expressing hA₃AR. CHO cells expressing hA₃ARs were incubated with 10 nM MRS5449 for different time intervals. Nonspecific binding was measured in the presence of 10 μ M MRS1220. The $t_{1/2}$ for association was found to be 53 min. Results are expressed as mean $\pm$ S.E. (n=3). D) Dissociation kinetics of MRS5449. The concentration of MRS5449 used in association and the dissociation experiments was 10 nM. Dissociation was initiated after 90 min by adding 10 μ M MRS1220. K_d was found to be 6.65 ± 0.55 nM. Results are expressed as mean $\pm$ S.E. (n=3).

Figure 6.

A) Test of known AR ligands (MRS1220, CI-IB-MECA, NECA and DPCPX). Competitive binding assay was performed using CHO cells expressing the hA₃AR incubated with 5 nM MRS5449 and increasing concentrations of the adenosine receptor ligands for 90 min at 37 °C. The K_i values are (nM): 3.46 ± 0.8 ; 27.7 ± 6.3 ; 149 ± 13 and 1320 ± 60 ; respectively. Results are expressed as mean $\pm$ S.E. (n=3). B) Detailed displacement curve of CI-IB-MECA using MRS5449 as a tracer. Competitive binding assay was performed using CHO cells expressing hA₃AR incubated with 5 nM MRS5449 and increasing concentrations of the CI-IB-MECA for 90 min at 37 °C. The displacement curve can be best fitted in a two-site model. Results are expressed as mean $\pm$ S.E. (n=2).

Figure 7. Hypothetical binding conformations at the hA₃AR model of MRS5449 (on the left) and compound **3** (on the right), obtained after molecular modeling studies. Ligand-receptor models are embedded in a solvated lipid bilayer, simulating the cell membrane, and are viewed through a cross-section of the plasma membrane.

Table 1

Binding affinity of a series of fluorescent AR antagonist derivatives at three subtypes of hARs.^a

Compound	A ₁	A _{2A}	A ₃
	K _i (nM) or % <i>Inhibition</i>	K _i (nM) or % <i>Inhibition</i>	K _i (nM) or % <i>Inhibition</i>
1	9500 ^b	920±110	780 ^b
2	(5±2%)	2480±420	2390±660
3	(7±5%)	460±60	720±70
4	(9±5%)	1750±470	5660±610
5	(0 %)	(30±6%)	9390±1730
6	(0 %)	6010±420	7090±370
7	3.5	1.2	35
8	170±40	51.0±10.0	33.5±10.1
9 ^c	87.0±24.0	73.0±8.0	6.4±2.5

^a All experiments were done on CHO (A₁ and A₃ARs) or HEK293 (A_{2A}AR) cells stably expressing one of three subtypes of hARs. The binding affinity for A₁, A_{2A} and A₃ARs was expressed as K_i values (n = 3–5, unless noted) and was determined by using agonist radioligands ([³H]R-PIA; [³H]CGS21680; or [¹²⁵I]I-AB-MECA; respectively), unless noted. A percent in parentheses refers to inhibition of radioligand binding at 10 μM, unless noted. Binding data for CGS15943 7 were taken from literature reports [5]. IC₅₀ values were converted to K_i values as reported [32].

^b duplicate determination.

^c 9, MRS5449.

Table 2

Inhibition of A₃AR binding of known AR agonists and antagonists using MRS5449 as a FCM tracer in whole cells.

Compound (selectivity)	hA ₃ AR radioligand binding, K _i (nM) ^a	FCM binding at hA ₃ AR, K _i (nM)
Agonists		
CPA	72	1490±230
CGS21680	67	1580±190
C1-IB-MECA	1.4	27.7±6.3
IB-MECA	1.8	10.9±1.9
NECA	25	149±13
Antagonists		
DPCPX	3980	1320±60
ZM241385	743	300±27
MRS1220	0.65	3.46±0.8
PSB-10 ^b	0.441	9.54±2.2
XAC	13.8	19.9±1.8
CGS15943	35	16.1±6.2
Theophylline	22,300	29,400±1400

^aK_i values for A₃AR binding affinity in cell membrane preparations using [¹²⁵I]I-AB-MECA are reported [5,14,33].

^bThe potency of PSB-10 was 4 nM in a functional assay.