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Novel Alexa Fluor-488 labeled antagonist of the A_{2A} adenosine receptor: Application to a fluorescence polarization-based receptor binding assay

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ABSTRACT

Fluorescence polarization (FP) assay has many advantages over the traditional radioreceptor binding studies. We developed an A_{2A} adenosine receptor (AR) FP assay using a newly synthesized fluorescent antagonist of the A_{2A}AR (MRS5346), a pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine derivative conjugated to the fluorescent dye Alexa Fluor-488. MRS5346 displayed a K_i value of 111 ± 16 nM in radioligand binding using [^{3}H]CGS21680 and membranes prepared from HEK293 cells stably expressing the human A_{2A}AR. In a cyclic AMP functional assay, MRS5346 was shown to be an A_{2A}AR antagonist. MRS5346 did not show any effect on A_1 and A_3 ARs in binding or the A_{2B}AR in a cyclic AMP assay at 10 μM. Its suitability as a fluorescent tracer was indicated in an initial observation of an FP signal following A_{2A}AR binding. The FP signal was optimal with 20 nM MRS5346 and 150 μg protein/mL HEK293 membranes. The association and dissociation kinetic parameters were readily determined using this FP assay. The K_d value of MRS5346 calculated from kinetic parameters was 16.5 ± 4.7 nM. In FP competition binding experiments using MRS5346 as a tracer, K_i values of known AR agonists and antagonists consistently agreed with K_i values from radioligand binding. Thus, this FP assay, which eliminates using radioisotopes, appears to be appropriate for both routine receptor binding and high-throughput screening with respect to speed of analysis, displaceable signal and precision. The approach used in the present study could be generally applicable to other GPCRs.
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1. Introduction

The A_{2A}AR is one of the four adenosine receptor (AR) subtypes of the G protein-coupled receptor (GPCR) family which mediates multiple physiological effects of extracellular adenosine, both in the central nervous system (CNS) and in peripheral tissues, activating several G protein-dependent and independent signaling pathways [1]. Increasing evidence relates selective A_{2A}AR ligands to applications in cardiac stress testing [2], neurodegenerative disorders such as Parkinson's disease [3,4] and inflammation [5], renewing the interest in these receptors, which are increasingly viewed as promising therapeutic targets [6].

GPCRs represent the single largest molecular target of therapeutic drugs and are also the most common focus in high-throughput screening assays designed to identify potential new drug candidates. A major fraction of these assays are now formatted as radioligand binding assays. Numerous agonists and antagonists have been developed by medicinal chemical methods either by empirical probing to establish a structure activity relationship (SAR) or in quantitative structure activity relationship (QSAR) studies [7]. Thus, many laboratories currently seek to identify novel leads for potent A_{2A}AR agonists and antagonists. Typically, to achieve this aim they use a radioligand-based binding assay and receptor-specific functional assays.

An alternative and potentially more versatile approach to measure affinity in receptor binding is fluorescence polarization (FP). FP offers several advantages in comparison to radioligand assays, such as low cost and avoiding problems like health hazards of radiation exposure and radioactive waste disposal. Furthermore,

Abbreviations: AF488, Alexa Fluor-488; CHO, Chinese hamster ovary; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS21680, 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamido-adenosine; DMEM, Dulbecco's Modified Eagle Medium; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FP, fluorescence polarization; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; HTS, high-throughput screening; [^{125}I]AB-MECA, [^{125}I]4-amino-3-iodobenzyl-5′-*N*-methylcarboxamidoadenosine; mP, millipolarization; MRS5346, 5-((2-(2-(4-(3-(5-amino-2-(furan-2-yl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl)propyl)phenoxy)acetamido)ethyl)-carbamoyl)-2-(6-amino-3-iminio-4,5-disulfonato-3*H*-xanthen-9-yl)benzoate; NECA, 5′-*N*-ethylcarboxamidoadenosine; SCH442416, 2-(2-furyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine; SCH58261, 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine; XAC, xanthine amine congener, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine; ZM241385, 4-[2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-a][1,3,5]triazin-5-yl-amino]ethylphenol.

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FP is faster and easier experimentally, involving only a homogenous measurement without filtration or other additional steps. Fluorescent ligands for various GPCRs including the ARs have been reported [8,9], but several analytical issues have hampered the development of receptor binding assays based on fluorescence intensity alone. These assays are usually heterogeneous and require a filtration step for the separation of the bound and unbound fractions of fluorescent ligand [10].

FP measurements are based on the assessment of the rotational motions of molecular species [9]. FP can be considered a competition between the molecular motion and the lifetime of fluorophores in solution. If linear polarized light is used to excite an ensemble of fluorophores, only those fluorophores that are aligned with the plane of polarization will be excited. There are two scenarios for the fluorescence emission. Provided the fluorescence lifetime of the excited fluorescent probe is much longer than the rotational correlation time of the molecule to which it is bound, e.g., when the fluorescent ligand is free in the solution, the molecules will randomize in solution during the emission process. As a result, the emitted light of the fluorescent probe will be depolarized. If the fluorescence lifetime of the fluorophore is much shorter than the rotational correlation time, e.g., when the fluorescent ligand is bound to a membrane receptor, the excited molecules will stay aligned during the process of emission, and the resulting emission will be polarized and detectable. This technique has been described as fast, sensitive, and inexpensive and is therefore a method of choice for high-throughput screening (HTS), although so far it has been applied to only a limited number of GPCRs mainly due to the difficulties in synthesizing an appropriate FP ligand [11,12].

Now with an X-ray structure of the human (h) A_{2A}AR available [13], it is possible to select in silico and rationally design many more potent antagonists of the receptor. Use of the 3D structure of the A_{2A}AR in theoretical ligand docking has also been demonstrated. It should be highly beneficial to use a fluorescence-based HTS assay to measure the binding affinity of the novel analogues at the A_{2A}AR. In this study, we have introduced a new fluorescent tracer (MRS5346; 5-((2-(2-(4-(3-(5-amino-2-(furan-2-yl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl)propyl)phenoxy)acetamido)-ethyl)-carbamoyl)-2-(6-amino-3-iminio-4,5-disulfonato-3*H*-xanthen-9-yl)benzoate, Fig. 1) for FP studies, which contains an Alexa Fluor (AF)488 moiety, and demonstrated its utility in pharmacological experiments.

2. Materials and methods

2.1. Materials

AF 488 carboxylic acid 2,3,5,6-tetrafluorophenyl ester was purchased from Invitrogen (Carlsbad, CA). [^{3}H]*R*-N^6-(2-phenylisopropyl)adenosine ([^{3}H]R-PIA, 42.6 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). [^{125}I]4-Amino-3-iodobenzyl-5'-N-methylcarboxamidoadenosine ([^{125}I]AB-MECA, 2200 Ci/mmol), [^{3}H]2-chloro-N^6-cyclopentyladenosine and [^{3}H]-2-[p-(2-carboxyethyl)phenylethylamino]-5′-N-ethylcarboxamidoadenosine ([^{3}H]CGS21680, 40.5 Ci/mmol) were purchased from PerkinElmer (Waltham, MA). DMEM/F12 medium and 1 M Tris–HCl (pH 7.5) were purchased from Mediatech, Inc. (Herndon, VA). Adenosine deaminase, theophylline, caffeine, XAC, Cl-IB-MECA and NECA were from Sigma (St. Louis, MO). CGS21680, SCH58261, SCH442416, ZM241385 and CPA were from Tocris (St. Louis, MO). The synthesis of MRS5346 will be described elsewhere (Kumar et al., manuscript in preparation). The final product was purified by semipreparative HPLC with a 250 mm × 10.0 mm RP-C18 reversed phase column (Phenomenex Inc., Torrance, CA). A mobile phase gradient of water:acetonitrile of 100:0 (v/v) to 70:30 in 25 min with a flow rate of 2 mL/min was used. Subsequent analytical HPLC of MRS5346 demonstrated purity of >98%. All other reagents were from standard resources and were of analytical grade.

2.2. Cell culture and membrane preparation

CHO cells stably expressing the hA_1, hA_{2A}, hA_{2B} or hA_3ARs and HEK293 cells expressing the hA_{2A}AR were grown in DMEM/F12 (1:1), 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin/streptomycin and DMEM, 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin/streptomycin, respectively. After harvest and homogenization, the cells were centrifuged at 200 × *g* for 10 min, and the pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.5), containing 10 mM $MgCl_2$ and 1 mM EDTA. The suspension was homogenized with an electric homogenizer for 10 s and was then re-centrifuged at 20,000 × *g* for 20 min at 4 °C. The resultant pellets were resuspended in buffer in the presence of 3 U/mL adenosine deaminase, and the suspension was stored at −80 °C until the binding experiments. The protein concentration was measured using the Bradford assay [14].

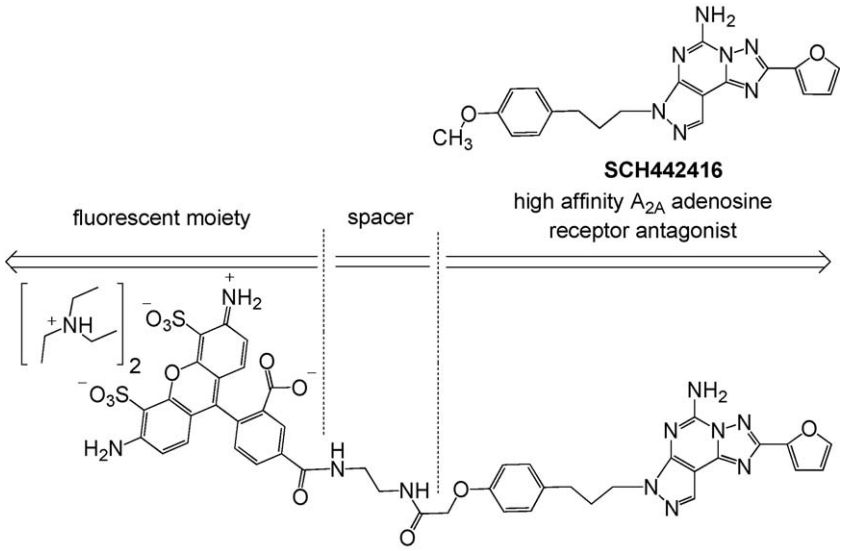

Fig. 1. The chemical structures of SCH442416 and its fluorescent derivative MRS5346.

2.3. Radioligand membrane binding assays

Radioligand binding assays at A_1, A_{2A}, and A_3 ARs were performed according to the procedures described previously [15]. Each tube in the binding assay contained 100 μL of membrane suspension (20 μg of protein), 50 μL of agonist radioligand, and 50 μL of increasing concentrations of the test ligands in Tris–HCl buffer (50 mM, pH 7.5) containing 10 mM $MgCl_2$. Non-specific binding was determined using a final concentration of 10 μM NECA, a non-specific agonist, diluted with the buffer. The mixtures were incubated at 25 °C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburg, MD). The radioactive agonists [^{3}H]R-PIA and [^{3}H]CGS21680 were used for the A_1 and A_{2A} assays, respectively, while [^{125}I]AB-MECA was used for the A_3AR assays. All of the filters were washed 3 times with Tris–HCl, pH 7.5. Filters for A_1 and A_{2A}AR binding were placed in scintillation vials containing 5 mL of hydrofluor scintillation buffer and counted using a TriCarb 2810 TR Liquid Scintillation Analyzer (PerkinElmer, Boston, MA). Filters for A_3AR binding were counted using a Packard Cobra II γ-counter (PerkinElmer, Boston, MA).

2.4. Cyclic AMP accumulation assay

CHO cells expressing the A_{2A} or A_{2B} AR were seeded in 24-well plates and incubated at 37 °C overnight. The following day the medium was removed and replaced with DMEM containing 50 mM HEPES, 10 μM rolipram, 3 U/mL adenosine deaminase, and increasing concentrations of agonists. 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.

2.5. FP binding assay for the A_{2A}AR

Assays were conducted in Costar 96-well black clear-bottom plates (Corning, Inc., Corning, NY). The binding buffer used in the FP measurement was the same as the buffer in the radioligand binding assay. Competition assays were performed with 50 μL AF 488 labeled ligand, always diluted from DMSO stock (final concentration of MRS5346 was 20 nM), 50 μL competitor (final concentrations 10 μM to 0.01 nM), and 100 μL membrane (150 μg/well) in the binding buffer for a total volume of 200 μL. Non-specific binding was measured in the presence of 4 μM of the non-selective AR agonist NECA. Binding conditions were similar to the radioligand binding (60 min incubation at 30 °C). The association kinetics of MRS5346 was measured at different time points. The dissociation kinetics was measured by adding 4 μM NECA at various time points after incubation of MRS5346 and the membrane preparations for 60 min. The FP signal was measured on a SpectraMax M5 microplate reader using SoftMax Pro5 software as described above (Molecular Devices, CA). According to both the literature and our measurement of MRS5346, the AF 488 absorption and emission peaks are at 495 and 519 nm. For FP measurements we used 480 nm excitation and 520 nm emission wavelengths because of a better separation.

2.6. Data analysis

Data analysis was performed with the PRISM Software, and K_i values were calculated using the Cheng–Prusoff equation [16]. The K_d for the A_{2A}AR-FP ligand binding was obtained by a kinetic on/off experiment. To determine binding kinetics parameters, we fit the 1-phase exponential association equation $y = y_{max}(1 - e^{kx})$ to the specific binding data. The variable K is the observed rate constant, K_{obs}, expressed per minute, which was then correlated with the concentration of the [FP] ligand to calculate K_{on} and K_{off} (dissociation rate constant). K_d was estimated through calculation from these parameters: $K_d = K_{off}/K_{on}$; $K_{on} = K_{obs} - K_{off}$.

3. Results

3.1. FP assay development and optimization

3.1.1. Choice of FP ligand

In considering the design of an appropriate FP ligand for the A_{2A}AR, first we had to choose a small ligand with a relatively high affinity at the receptor that was suitable for derivatization as a functionalized congener [17]. We selected the antagonist SCH442416, which has high affinity and selectivity for the A_{2A}AR [18], as a lead molecule. The strategy for selection of the site for chain extension and fluorophore conjugation is based on the X-ray structure of the A_{2A}AR and will be described elsewhere. The second step was to find an appropriate fluorophore for FP. There are several important considerations in this selection, but the most relevant is the fluorescent lifetime. The fluorescence lifetime refers to the average time that the molecule stays in its excited state before emitting a photon. If the fluorophore has a lifetime that is too short, it does not have sufficient time for rotation, which was why we obtained a low polarization value both with the competitor ligand bound to the receptor and in its free state. The fluorophore AF 488 has a 4.1 ns lifetime and is therefore suitable for FP measurement. It is important to note that the fluorescent probes must be constructed to give a maximum polarization signal when bound to the receptor. Therefore, the linker between the fluorophore and the ligand must be relatively short and rigid. The chemical approaches to achieving this aim, which were based on the knowledge of molecular recognition at the receptor orthosteric binding site, will be described elsewhere.

The A_{2A}AR binding affinity of the FP ligand was measured in the radioligand binding assay using [^{3}H]CGS21680 as a radioligand (Fig. 2). It was found that the K_i value of MRS5346 for the A_{2A}AR was 111 ± 16 nM. The affinity of MRS5346 at the two other adenosine receptors was very low; at A_1 and A_3 ARs, the degree of inhibition of radioligand binding at 10 μM was 4.6 ± 1.9% and 3.8 ± 2.1%, respectively. According to its fluorescent properties and its binding affinity to the A_{2A}AR, MRS5346 seemed to be an acceptable ligand for the polarization measurement.

In functional assays, it was shown that MRS5346 inhibited cyclic AMP accumulation in CHO cells expressing the A_{2A}AR induced by the A_{2A}AR agonist CGS21680. At 1 μM, MRS5346

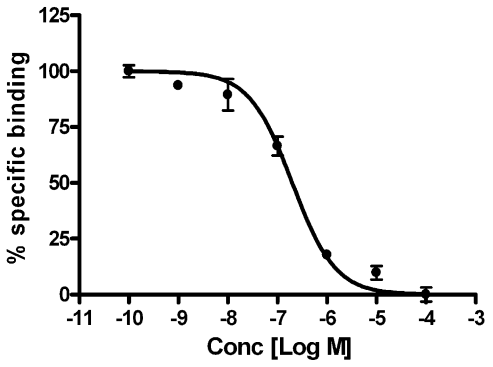

Fig. 2. Displacement by MRS5346 of [^{3}H]CGS21680 binding to membrane preparations from HEK293 cells stably expressing the human A_{2A}AR. Binding was performed using 10 nM [^{3}H]CGS21680, 20 μg/well proteins and increasing concentrations of MRS5346. Results are expressed as mean ± S.E. (n = 3).

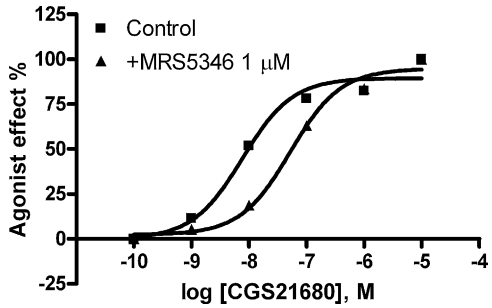

Fig. 3. Effect of the fluorescent antagonist MRS5346 on the A_{2A}AR agonist CGS21680-induced cyclic AMP accumulation in CHO cells expressing the A_{2A}AR. The experiment was performed using 10 μM rolipram, 3 U/mL adenosine deaminase, and increasing concentrations of agonists. At 1 μM MRS5346 shifted the concentration–response curve of CGS21680 to the right by about 6-fold.

shifted the concentration-response curve of CGS21680 (K_i 27 nM) to the right about 6-fold (Fig. 3). At 10 μM, MRS5346 did not inhibit NECA-induced cyclic AMP accumulation in CHO cells expressing the A_{2B} AR.

3.1.2. Determination of concentration of membrane preparation and FP ligand concentration to be used in the assay

The first step in the development of an FP assay was to determine the appropriate concentrations of the membrane preparation. Using membranes prepared from HEK293 cells stably expressing the A_{2A}AR, we measured the optimal concentration of the membrane preparation for the FP signal. As seen in Fig. 4, the FP signal was proportional to the concentration of the A_{2A}AR membranes in the suspension with a good separation between specific and non-specific binding. The non-specific binding was determined in the presence of 4 μM NECA. To balance the opposing aims of minimizing ligand depletion while maximizing the signal to noise ratio, we chose the 150 μg/well protein concentration for all future studies. At this concentration MRS5346 showed significant increases in the FP signal upon binding to the A_{2A}AR. Also, it was noted that at the concentration 20 nM, MRS5346 had the largest mP (millipolarization) window (difference in mP units before and after polarization) (Fig. 5). Lower concentrations (2 and 10 nM) produced reduced FP signal (data not shown). Therefore, we chose the 20 nM concentration of MRS5346 to be used in subsequent studies.

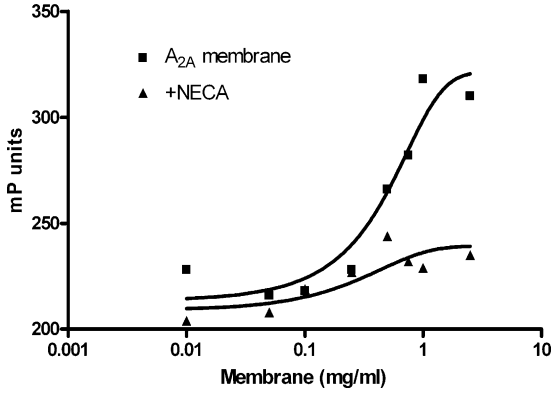

Fig. 4. Optimization of the concentration of membrane preparations from HEK293 cells stably expressing the human A_{2A}AR. A fixed concentration of MRS5346 (20 nM) and increasing concentrations of the membranes were used. After incubation for 60 min at 30 °C, the FP values (mP units) were determined. Results were from a representative experiment from three separate experiments of similar results, each performed in duplicate. To measure the non-specific binding we used 4 μM NECA.

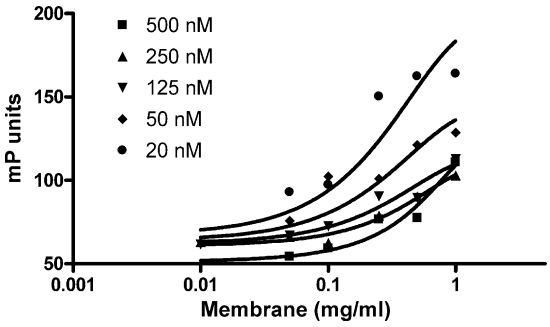

Fig. 5. Determination of the appropriate concentration of the FP ligand. Various concentrations of MRS5346 were used against various concentrations of membrane preparation from HEK293 cells stably expressing the human A_{2A}AR. Results were from one experiment performed in duplicate representative of three separate experiments of similar results performed in duplicate. The non-specific binding was determined using 4 μM NECA.

3.1.3. Determination of K_d

In contrast with the classical radioligand-based saturation experiments, the FP saturation binding assay is potentially complicated because of the presence of bound and unbound ligand in the detected signal. Therefore, we measured the K_d of MRS5346 using the association and dissociation rates of the ligand to the receptor. With this measurement we obtained the on- and off-rates for the ligand binding to the receptor, and using these rates the K_d value could be determined. Fig. 6 shows the time which is needed to reach equilibrium (A) and then the dissociation curve (B) using 4 μM NECA. The K_d value calculated using the FP based

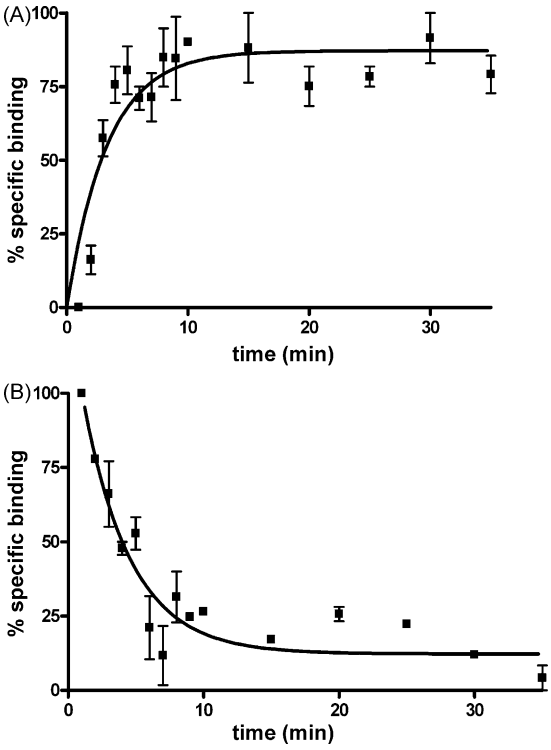

Fig. 6. Kinetic measurements of MRS5346 in binding to membrane preparation from HEK293 cells stably expressing the human A_{2A}AR. In the association experiment (A) we determined the on-rate of the FP ligand (20 nM) using 150 μg/well membranes. The FP was measured in every min for 10 min and after every 5 min for 30 min. In the dissociation experiment (B) we determined the off-rate using 4 μM NECA continuing the association experiment in the 30 min. Results are expressed as mean ± S.E. (n = 3). The specific binding measured was typically 100–150 mP units.

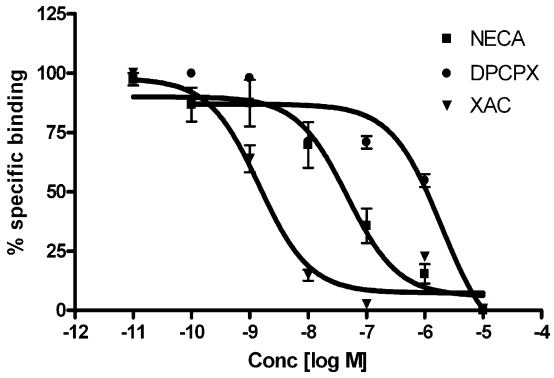

Fig. 7. FP competition binding experiment with NECA, XAC and DPCPX. Binding was performed using 20 nM MRS5346, 150 μg/well membranes and increasing concentration of the NECA, XAC and DPCPX in a final assay volume of 200 μL. The K_i values (nM) are: NECA, 21.2 ± 3.4; XAC, 3.8 ± 0.9; and DPCPX, 630 ± 170 nM. The specific binding measured was typically 100–150 mP units. Results are expressed as mean ± S.E. (n = 3).

kinetic data was 16.5 ± 4.7 nM (n = 3), which roughly matched the K_i value determined from the competition experiment using [^{3}H]CGS21680 (111 ± 16 nM).

3.2. Screening of known A_{2A}AR ligands

Radioligand-based receptor binding assays for pharmacologically characterizing receptor interactions are more common and well-characterized techniques than fluorescent methods. However, experiments using radioligands have several disadvantages (cost, health hazard, and waste disposal problem), even though they are widely used because of their reliability. In order to replace a radioligand binding assay with a new technique, it will be necessary to demonstrate that the new method is equally reliable and useful for a wide range of the ligands with different chemical structures. Therefore, we tested several agonists and antagonists of the A_{2A}AR with a wide range of affinities and included both selective and general AR ligands. We chose some selective A_{2A}AR ligands such as agonist CGS21680 and the antagonists ZM241385 and SCH58261. We also tested some non-selective ligands, such as the nucleoside agonist NECA and the xanthine antagonists caffeine, theophylline, and XAC (xanthine amine congener). We also chose an A_1AR agonist CPA and an A_3AR agonist Cl-IB-MECA, which are relatively weak at the A_{2A}AR. A few representative ligands used were shown in Fig. 7, and the K_i values of all compounds tested were listed in Table 1. As is evident in Table 1, all of the K_i values from the FP binding assay closely matched the reported values from the radioligand binding assay. The correlation coefficient for FP data in comparison to radioligand binding affinities in Table 1 is 0.81.

Table 1
Comparison of the K_i values (nM) determined using fluorescent polarization (FP) and radioligand binding assays.

	K_i (nM)	
	FP	Radioligand binding [1,27–29]
Agonists		
NECA	21.2 ± 3.4	20
CGS21680	27.3 ± 7.2	27
CPA	814 ± 170	794
Cl-IB-MECA	3720 ± 870	5360
Antagonists		
XAC	3.7 ± 0.87	1
SCH442416	11.1 ± 1.14	4.1
SCH58261	1.9 ± 0.66	0.6
ZM241385	1.3 ± 0.86	1.6
Theophylline	2030 ± 1790	1700
Caffeine	2480 ± 450	9560
DPCPX	630 ± 170	129

4. Discussion

The present study demonstrated that the novel fluorescent antagonist, MRS5346, can be successfully used as a tool in the FP A_{2A}AR binding assay, which represents the first example in the AR field. This FP ligand should be applicable in many areas for studies related to the A_{2A}AR, including kinetic analysis and tests of ligand binding affinity. MRS5346 appears to be suitable for both regular binding assay and high-throughput screening. The FP assay should be applicable to both the study of othosteric and allosteric sites on the A_{2A}AR [19]. The dissociation kinetics is particularly useful for high-throughput screening of allosteric modulators which has been laborious using radiolidgands [19]. With the assistance of structure of the recently crystallized A_{2A}AR, it is possible that more ligands targeting both the orthosteric and allosteric sites will be designed and subsequently tested with a binding assay. Thus, the FP assay using MRS5346 developed in the present study should find wide application in pharmacological and medicinal chemical studies.

Although FP assays have been applied to the study of several members of GPCRs, such as melanocortin-4 receptor [20], 5-HT receptors [21], muscarinic receptors [22], the application of FP to ARs has not yet been successful mainly due to the difficulty in the design of an appropriate FP ligand. Earlier studies were also limited by the lack of sensitive fluorescence detectors in addition to the suitable fluorescent probes. Many attempts have been made at conjugating fluorescent molecules to both known and novel receptor ligands in the hope of identifying proper fluorescent ligands to replace radioligands [8,23–26]. For example, a Cyanine5 (Cy5) labeled fluorescent agonist MRS5218 synthesized earlier [26] showed high affinity for A_3AR and good fluorescent intensity, but the fluorophore was not suitable as a FP ligand possibly due to its short lifetime (0.1 ns). A multivalent fluorescent dendrimer derivative of an adenosine agonist labeled with AF 488 was shown to bind to the hA_3AR expressed in CHO cells with nanomolar affinity, but this conjugate would not be suitable for an FP assay due to the flexibility of the polymeric carrier moiety [30].

Radioligand binding to GPCRs is one of the most common assays used in the study of GPCRS. Despite its popularity, regulatory pressure to reduce the use of radioactivity in screening laboratories and several drawbacks, including high cost, short shelf-life and potential health and disposal harzards, has led to considerable effort to develop non-radioactive alternatives. Compared to the commonly used radioligand binding assay, this FP measurement provides a rapid and convenient means to determine the rotational rate of a fluorophore as well as information about ligand receptor binding property with comparable accuracy to that obtained using radioligand binding assays. This sensitive and homogenous assay format could potentially be used for both regular receptor binding and high-throughput screening. The approach used in this assay should also be applicable to other GPCRs. According to the results of this study, the assay using MRS5346 for FP at the A_{2A}AR in a binding competition mode could be widely applied to assessing the affinity of new ligands.

It is noted that in the FP assay a relatively high membrane protein concentration is needed to maximize the window of polarized fluorescence in comparison to the non-polarized fluorescence, which is typical for this and other FP assays [20]. We are aware that there is a 7-fold difference in the K_i value measured in the [^{3}H]CGS21680 displacement experiment and the

K_d value calculated from kinetic parameters determined with the FP ligand MRS5346. A maximally 3–5-fold difference in K_i values was also observed for a few of other ligands as listed in Table 1. However, it does not seem to be surprising considering that the experiment conditions of the two assays are not identical and the fact that CGS21680 is an agonist while MRS5346 is an antagonist. It has been shown previously [28] that K_i values may differ somewhat (typically <10-fold) under slightly different experimental conditions especially by using agonist versus antagonist radioligands. Also, it should noted that the direct measurement of the affinity of MRS5346 in the FP displacement assay is not feasible.

Thus, the present study represents the first successful example of the application of FP assay in the A_{2A}AR binding studies. As there are no reports for application of FP assay to other subtypes of ARs yet, the development of novel FP ligands for other AR subtypes based on the rationale used in the present study should also be possible.

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