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Allosteric modulators of human A_{2B} adenosine receptor

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ABSTRACT

Background: Among adenosine receptors (ARs) the A_{2B} subtype exhibits low affinity for the endogenous agonist compared with the A_1, A_{2A}, and A_3 subtypes and is therefore activated when concentrations of adenosine increase to a large extent following tissue damages (e.g. ischemia, inflammation). For this reason, A_{2B} AR represents an important pharmacological target.
Methods: We evaluated seven 1-benzyl-3-ketoindole derivatives (**7**–**9**) for their ability to act as positive or negative allosteric modulators of human A_{2B} AR through binding and functional assays using CHO cells expressing human A_1, A_{2A}, A_{2B}, and A_3 ARs.
Results: The investigated compounds behaved as specific positive or negative allosteric modulators of human A_{2B} AR depending on small differences in their structures. The positive allosteric modulators **7a**,**b** and **8a** increased agonist efficacy without any effect on agonist potency. The negative allosteric modulators **8b**,**c** and **9a**,**b** reduced agonist potency and efficacy.
Conclusions: A number of 1-benzyl-3-ketoindole derivatives were pharmacologically characterized as selective positive (**7a**,**b**) or negative (**8c**, **9a**,**b**) allosteric modulators of human A_{2B} AR.
General significance: The 1-benzyl-3-ketoindole derivatives **7**–**9** acting as positive or negative allosteric modulators of human A_{2B} AR represent new pharmacological tools useful for the development of therapeutic agents to treat pathological conditions related to an altered functionality of A_{2B} AR.

1. Introduction

Adenosine acts as an agonist of four subtypes of G protein-coupled receptors (GPCRs), the A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (ARs) [1–3]. Among ARs, the A_{2B} subtype displays the lowest affinity for its endogenous agonist and is therefore activated when adenosine is released at high levels as one of the consequences of tissue damage [4,5]. A_{2B} AR is expressed in the gastrointestinal tract, bladder, lung, mast cells, eye, adipose tissue, brain, kidney, liver, and other tissues [2,6]. Activation of A_{2B} AR triggers the stimulation of adenylate cyclase and phospholipase C by coupling to Gs and $Gq_{/11}$ proteins, respectively, with a subsequent increase of intracellular cAMP and calcium ion levels [7]. A_{2B} AR is involved in several physiological and pathological processes, including glucose metabolism [8], angiogenesis induction [9,10], growth of some tumors [11], intestinal inflammation [12,13], myocardial ischemia [14,15], acute lung and kidney injury [16,17].

A_{2B} AR is the least well-characterized subtype in the AR family, partly due to the scarcity of specific ligands [3,18–20]. The only class of potent and selective A_{2B} AR agonists thus far disclosed is represented by some phenylpyridinesulfanylacetamide derivatives whose leading exponent, BAY 60-6583 (**1** in Chart 1), is currently being investigated for the treatment of atherosclerosis and cardiac disorders [15,20,21]. BAY 60-6583 has also been reported to reduce inflammation after ventilator-induced lung injury [22] and to modulate myocardial adaptation to ischemia [14]. Several selective A_{2B} AR antagonists provided with high potency and selectivity have been identified as potential therapeutic agents for the treatment of diabetic retinopathy and cancer [23,24], colitis [25,26], and asthma [27–29]. The classes of A_{2B} AR antagonists described to date [3,18–20,28] include pyrrolopyrimidines (**2**) [30], pyrazolotriazolopyrimidines (**3**) [31], 2-aminopyrazines (**4**) [32], xanthines (**5**) [33], and triazinobenzimidazolones (**6**) [34] (**2**–**6** in Chart 1). Until today, no allosteric modulators of A_{2B} AR have been described in the literature [35–37]. Recently, netrin-1, an 85 kDa protein acting as migration and adhesion cue in the developing central

Abbreviations: ADA, adenosine deaminase; AR, adenosine receptor; cAMP, 3′,5′-cyclic adenosine monophosphate; BAY 60-6583, 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide; CHO, Chinese hamster ovary; DMAP, 4-dimethylaminopyridine; DMEM, Dulbecco's Modified Eagle Medium; GPCR, G protein-coupled receptor; [^{35}S]GTPγS, [^{35}S]guanosine 5′-O-[gamma-thio]triphosphate; [^{3}H]MRS 1754, [^{3}H]N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)-phenoxy]acetamide; [^{3}H]NECA, [^{3}H]5′-*N*-ethylcarboxamideadenosine; Ro 20-1724, 4-(3-butoxy-4-methoxyphenyl) methyl-2-imidazolidone

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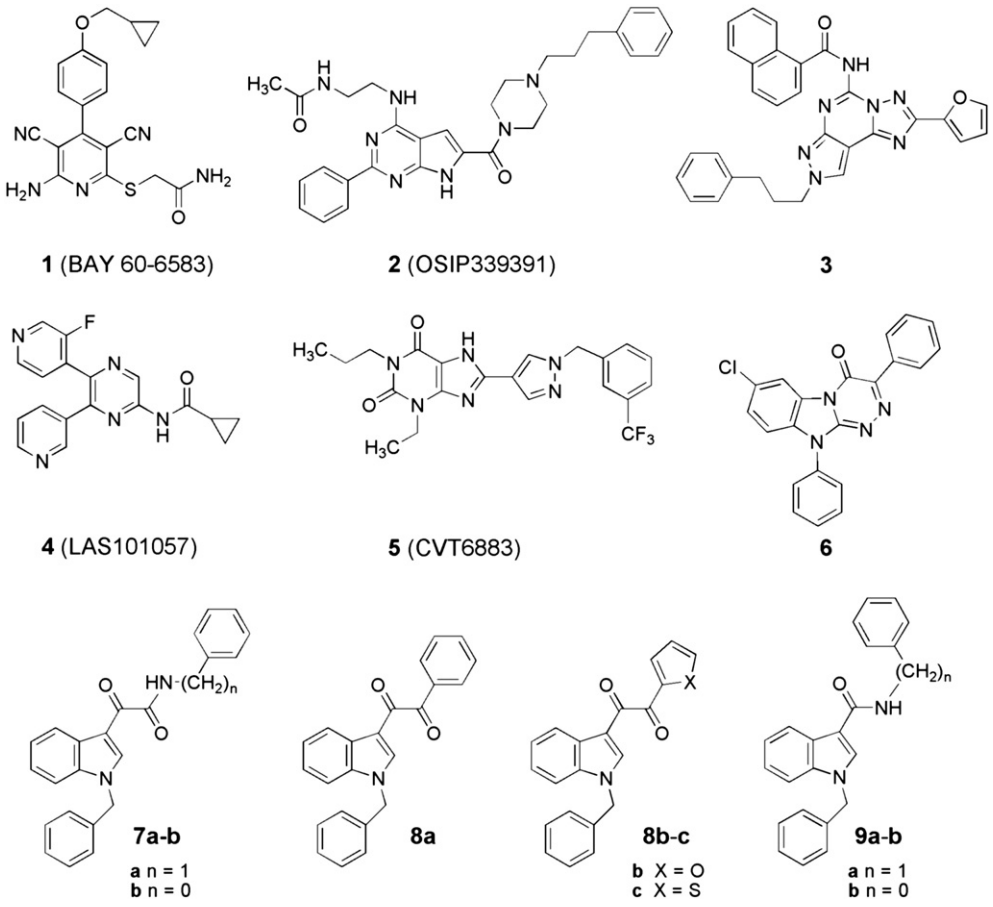

Chart 1. The structures of the compounds reported in the literature to be A_{2B} AR agonists (**1**) and antagonists (**2**–**6**), and of the new 1-benzyl-3-ketoindole derivatives **7a**,**b**, **8a**–**c**, **9a**,**b**.

nervous system and in a number of non-neural tissues, has been demonstrated to attenuate neutrophil transmigration and experimental colitis by modulating A_{2B} AR signaling [12,13]. However, development of this macromolecule as a drug is hampered by high costs required for its production and lack of oral bioavailability.

Recently, we synthesized a few 1-benzyl-3-ketoindole derivatives (**7**–**9** in Chart 1) designed as potential AR antagonists that, surprisingly, showed to be modulators of human A_{2B} AR [38]. Here, we describe the detailed biological characterization of compounds **7**–**9**, based on binding and functional assays using CHO cells expressing human A_1, A_{2A}, A_{2B}, and A_3 ARs, revealing that these compounds behave as positive (**7a**,**b** and **8a**) or negative (**8c** and **9a**,**b**) allosteric modulators of A_{2B} AR.

2. Experimental

2.1. Investigated compounds

Compounds **7a**,**b** and **8a**–**c** were obtained by simple and high yield synthetic procedures reported previously [38]. Compounds **9a**,**b** are commercially available (Bionet). Purity of tested compounds is ≥95% (combustion analysis).

2.2. Biology

2.2.1. Adenosine receptor binding assays. materials

[^{3}H]NECA and [^{35}S]GTPγS were obtained from DuPont-NEN (Boston, MA); [^{3}H]MRS 1754 was purchased from Scopus Research BV (Veenendaal, Netherlands). Adenosine deaminase (ADA) was obtained from Roche Diagnostics S.p.A. (Monza, Italy). BAY 60-6583 was purchased from Tocris bioscience (Bristol, UK). All other reagents were obtained from standard commercial sources and were of the highest commercially available grade. CHO cells stably expressing human A_1, A_{2A}, A_{2B}, and A_3 ARs were kindly supplied by Prof. K. N. Klotz, Wurzburg University, Germany [39].

2.2.2. Human A_1, A_{2A}, and A_3 adenosine receptor binding assays

The binding affinity of the investigated compounds towards human A_1, A_{2A}, and A_3 ARs, expressed in CHO cells, was evaluated as previously described [40].

2.2.3. Human A_{2B} adenosine receptor binding assays

2.2.3.1. Equilibrium binding assays. Membrane preparation was performed as previously described, with minor modifications [41]. Briefly, confluent monolayers of CHO cells expressing human A_{2B} AR were washed with PBS followed by ice-cold buffer T_1 (10 mM HEPES, 10 mM EDTA, pH 7.4). The cells were homogenized and centrifuged at 48,000 *g*. The pellets were washed twice with buffer T_2 (10 mM HEPES, 1 mM EDTA, pH 7.4) and were resuspended in buffer T_3 (50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4). The membranes were centrifuged at 10,000 *g* for 45 min at 4 °C, and the pellets were frozen in aliquots at −80 °C.

Binding experiments with [^{3}H]MRS 1754 were performed as essentially described, with minor modification [42]. Briefly, aliquots of cell membranes (50 μg), were incubated 60 min at room temperature with 0.7 nM [^{3}H]MRS 1754 in a final volume of 1 mL of buffer T_4 (50 mM Tris–HCl, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, pH 7.4) with the addition of 0.0005% Tween 20, 0.5% albumin from bovine serum, 1 mM DL-dithiothreitol and 2 units/mL ADA.

For competition binding experiments, different concentrations of the agonist NECA (1 nM–10 μM) were added to the incubation medium in the absence or the presence of 10 μM compound **7b**. Non-specific binding was determined in the presence of 100 μM NECA. Incubations were terminated by rapid filtration through GF/C glass fiber filters and the bound radioactivity was washed three times with 4 mL of buffer T_4. The radioactivity was measured by liquid scintillation spectrometry.

Equilibrium binding assays with [^{3}H]NECA were performed as previously described, with some modifications [43]. Briefly, aliquots of cell membranes (25 μg), were incubated 180 min at room temperature with 30 nM [^{3}H]NECA in a final volume of 1 mL of buffer T_4 with the addition of 0.0005% Tween 20, 0.5% albumin from bovine serum, 1 mM DL-dithiothreitol and 2 units/mL ADA.

For competition binding experiments, different concentrations of agonist BAY 60-6583 (0.001 nM–2.5 μM) were added to the incubation medium in the absence (control) or presence of 10 μM of the tested compound (**7b** or **9b**). Non-specific binding was determined in the presence of 100 μM NECA. Incubations were terminated by rapid filtration through GF/C glass fiber filters and the bound radioactivity was washed three times with 4 mL of buffer T_4. Radioactivity was measured by liquid scintillation spectrometry.

2.2.3.2. Dissociation kinetic binding assays. Dissociation binding experiments were performed by preincubating membrane aliquots from CHO cells expressing human A_{2B} AR containing 25 μg proteins with 30 nM [^{3}H]NECA for 180 min at room temperature in a final volume of 1 mL of buffer T_4 with the addition of 0.0005% Tween 20, 0.5% albumin from bovine serum, 1 mM DL-dithiothreitol and 2 units/mL ADA. Dissociation was initiated by addition of 10 μM NECA in the absence (control) or in the presence of the tested compound (**7b** or **9b**) and determined at different time points. Non-specific binding in the presence of tested compound was also determined in parallel. Incubations were terminated by rapid filtration through GF/C glass fiber filters and the bound radioactivity was washed three times with 4 mL of buffer T_4. Radioactivity was measured by liquid scintillation spectrometry.

2.3. cAMP level measurement in human A_1, A_{2A}, A_{2B}, and A_3 AR-transfected CHO cells

Intracellular cAMP levels were measured using a competitive protein binding method [44]. CHO cells, expressing recombinant human ARs, were harvested by trypsinization. After centrifugation and resuspension in medium, cells (~30,000) were plated in 24-well plates in 0.5 mL of medium. After 24 h, the medium was removed, and the cells were incubated at 37 °C for 15 min with 0.5 mL of Dulbecco's Modified Eagle Medium (DMEM) in the presence of 1 unit/mL ADA and the phosphodiesterase inhibitor Ro 20-1724 (20 μM). The antagonist profile of the compounds towards A_{2A} and A_{2B} ARs was evaluated by assessing their ability to modulate NECA-mediated accumulation of cAMP. The antagonist profile of the compounds towards A_1 and A_3 ARs was evaluated by assessing their ability to counteract the NECA-mediated inhibition of cAMP accumulation in the presence of 1 μM forskolin as non-selective adenylate cyclase activator. Cells were incubated in the reaction medium (15 min at 37 °C) with different concentrations (1 nM–10 μM) of the tested compounds and then were treated with the agonist. Following incubation, the reaction was terminated by the removal of the medium and the addition of 0.4 N HCl. After 30 min, lysates were neutralized with 4 N KOH, and the suspension was centrifuged at 800 g for 5 min. For the determination of cAMP production, bovine adrenal cAMP binding protein was incubated with [^{3}H] cAMP (2 nM) and 50 μL of cell lysate or cAMP standard (0–48 pmol) at 0 °C for 150 min in a total volume of 300 μL. The bound radioactivity was separated by rapid filtration through GF/C glass fiber filters and washed twice with 4 mL 50 mM Tris–HCl, pH 7.4. Radioactivity was measured by liquid scintillation spectrometry.

2.4. Characterization of compounds as allosteric modulators of the human A_{2B} AR in functional response

To evaluate the allosteric modulatory activity of the compounds towards AR subtypes, cells were incubated with the agonist NECA at a concentration corresponding to its EC_{50} value (50 nM for A_{2A}, 100 nM for A_{2B} and 10 nM for A_1 and A_3 ARs) in the absence or in the presence of 10 μM of the tested compound. Each compound was also tested alone, in both transfected and wild-type CHO cells, to evaluate its agonism. The activity of compounds **7a**, **7b** and **8a** as positive allosteric modulators or compound **9b** as negative allosteric modulator of A_{2B} AR was evaluated as follows: A_{2B} AR-transfected CHO cells were treated with different compound concentrations in the presence of 100 nM NECA or 1 μM adenosine or 10 nM BAY 60-6583, and then the intracellular cAMP levels were determined, as described above. Furthermore, the modulatory activity of each compound on agonist potency and intrinsic activity was evaluated incubating cells with different agonist concentrations, ranging from 1 nM to 1 μM, in the absence or the presence of three different concentrations of the tested compound.

2.5. [^{35}S]GTPγS binding assays in human A_{2B} AR-transfected CHO cells

[^{35}S]GTPγS binding assays on membranes from CHO cells stably expressing human A_{2B} AR were performed as previously described with some modifications [45]. Briefly, cell membranes (10 μg) were incubated in assay buffer T_5 (50 mM Tris–HCl, 1 mM EDTA, 1 mM $MgCl_2$, 100 mM NaCl, 1 mM DL-dithiothreitol, 0.0005% Tween 20 and 0.5% albumin from bovine serum) in the presence of 2 units/mL ADA, 1 μM GDP and different concentrations of agonist BAY 60-6583, in the absence or presence of the tested compound (**7b** or **9b**). Binding was initiated by the addition of 0.2 nM [^{35}S]GTPγS and incubated for 30 min at 25 °C. Non-specific binding was measured in the presence of 10 μM unlabelled GTPγS. The reaction was stopped by rapid filtration and the plate was washed twice with 200 μL of buffer T_4. Radioactivity was measured by liquid scintillation spectrometry.

2.6. Data analysis

All binding and functional data were analyzed using the nonlinear regression curve fitting program GraphPad, version 5.0. EC_{50}, IC_{50} and K_i values were directly obtained from the concentration–response curves. Statistical analyses of binding data and functional assays were performed using Student's *t*-test. All values obtained are mean values of at least three different experiments each performed in duplicate.

3. Results and discussion

As mentioned, we have recently disclosed a new series of 1-benzyl-3-ketoindole derivatives (**7**–**9** in Chart 1) behaving as modulators of human A_{2B} AR [38]. None of the compounds displayed any significant binding affinity for A_1, A_{2A}, and A_3 ARs (K_i > 10,000 nM), with the exception of **8a** and **8b** which showed submicromolar affinity for A_1 AR (**8a** K_i 161.5 nM; **8b** K_i 343.0 nM) [38].

Herein, we describe the detailed pharmacological characterization of these compounds demonstrating that they interact with A_{2B} AR as positive (**7a**,**b** and **8a**) or negative (**8b**,**c** and **9a**,**b**) allosteric modulators, the former by enhancing efficacy of agonists, the latter by decreasing either efficacy and potency of agonists.

In designing and interpreting our experiments, we took into account what is currently known about allosteric modulators of GPCRs [46,47]. These compounds bind to the receptor at a site that is topographically distinct from the orthosteric site, leading to conformational changes in the receptor. As a consequence, the recognition and/or transduction properties of the receptor can be altered

Table 1
Effects of compounds **7**–**9** on cAMP production in CHO cells expressing human A_1, A_{2A}, A_{2B} and A_3 AR[a].

	% cAMP inhibition (vs forskolin, 100%)[b]				% cAMP production (vs agonist maximal effect, 100%)[c]			
	A_1 AR		A_3 AR		A_{2A} AR		A_{2B} AR	
cpd	Alone	+NECA	Alone	+NECA	Alone	+NECA	Alone	+NECA
7a	10.2 ± 1.1	51.2 ± 4.3	8.1 ± 0.4	51.1 ± 3.6	2.0 ± 0.1	58.2 ± 3.6	5.2 ± 1.0	75.9 ± 0.4*
7b	9.3 ± 1.1	57.4 ± 4.6	8.4 ± 0.2	54.2 ± 4.1	1.1 ± 0.1	58.4 ± 3.3	6.2 ± 1.3	83.6 ± 4.4*
8a	4.0 ± 0.2	26.2 ± 1.9	0 ± 0	53.1 ± 2.9	1.2 ± 0.2	53.3 ± 4.2	8.3 ± 1.2	127.0 ± 5.7***
8b	0 ± 0	24.3 ± 2.1	1.0 ± 0.9	50.0 ± 3.3	1.0 ± 0.8	48.3 ± 3.6	9.2 ± 1.2	1.0 ± 0.9
8c	0 ± 0	53.1 ± 4.4	0 ± 0	51.5 ± 3.0	1.0 ± 0.9	58.2 ± 4.6	8.4 ± 1.3	2.5 ± 1.1
9a	3.0 ± 0.1	45.4 ± 3.9	7.2 ± 0.4	56.2 ± 4.1	1.2 ± 0.9	53.1 ± 3.0	8.1 ± 1.2	1.2 ± 1.0
9b	0 ± 0	57.2 ± 5.1	8.3 ± 0.6	59.2 ± 4.2	1.1 ± 1.0	54.0 ± 4.1	7.2 ± 1.1	5.0 ± 2.0

[a] The effect of each compound (10 μM) was evaluated on cAMP production in CHO cells expressing human A_1, A_{2A}, A_{2B} and A_3 ARs (see biological section). Each compound was tested alone or in the presence of an EC_{50} concentration of NECA (50 nM for A_{2A}, 100 nM for A_{2B} and 10 nM for A_1 and A_3 ARs).
[b] Data are expressed as percentage inhibition of cAMP production versus forskolin (1 μM), set to 100%.
[c] Data are expressed as percentage of cAMP production versus agonist maximal effect (100%). All data represent the mean ± SEM of at least three different experiments each performed in duplicate.
* $p < 0.05$ vs agonist alone.
*** $p < 0.001$ vs agonist alone.

in either a positive or negative direction. Specifically, allosteric modulators modify one or more of the following pharmacodynamic parameters: ligand potency at the orthosteric site, agonist efficacy, kinetics of ligand binding to the orthosteric site, functional/conformational states of the receptor, specific intracellular signaling pathways.

*3.1. Pharmacological profile of compounds **7a**,**b** and **8a** as positive allosteric modulators of A_{2B} AR*

3.1.1. cAMP functional assays

The efficacy profiles of compounds **7a**,**b** and **8a** towards ARs were evaluated in functional assays by measuring the ability of these

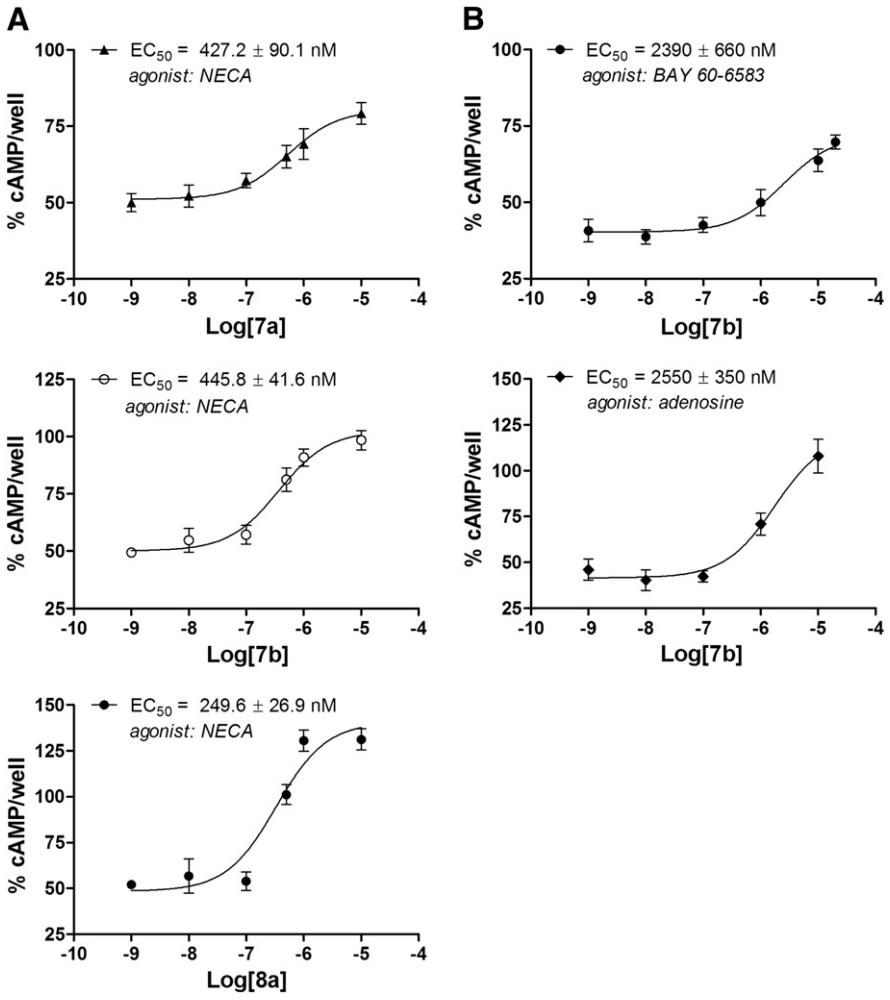

Fig. 1. Effects of **7a**, **7b** and **8a** on NECA-mediated cAMP accumulation (A), and of **7b** on BAY 60-6583 or adenosine-mediated cAMP accumulation (B) in human A_{2B} AR-transfected CHO cells. CHO cells were treated with an EC_{50} NECA concentration (100 nM) in the absence or presence of different concentrations of the tested compound (1 nM–10 μM). After 15 min incubation, the reaction was stopped and the intracellular cAMP levels were quantified. The data are expressed as the percentage of cAMP/well versus the maximal NECA effect, which was set to 100%, and represent the mean ± SEM of at least three different experiments. Each experiment was performed in duplicate.

compounds to modulate cAMP levels, either alone or in the presence of the agonist NECA, in human A_1, A_{2A}, A_{2B}, and A_3 AR-transfected CHO cells (Table 1). When tested alone at a 10 μM concentration, the compounds did not significantly increase the cAMP levels, thus demonstrating lack of intrinsic agonist activity at any of the ARs.

In the presence of an EC_{50} concentration of the agonist NECA, none of the compounds was able to modulate the cAMP levels in the A_1, A_{2A}, and A_3 AR-transfected CHO cells, with the exception of **8a**, which acted as A_1 AR antagonist [38].

In the assay involving A_{2B} AR, **7a**,**b** and **8a** potentiated the effects of NECA, suggesting that these compounds may behave as positive modulators of A_{2B} AR.

When functional experiments were performed on wild-type CHO cells that did not express ARs, the compounds did not show any influence on cAMP levels (data not shown), suggesting that these compounds enhance the NECA effects mediated by A_{2B} AR by specifically interacting with this receptor subtype. Moreover, any direct interaction of the compounds with G_s proteins could be safely excluded, as they did not exhibit any functional effect in CHO cells expressing A_{2A} AR, that is similarly coupled to G_s proteins [7].

The potencies of **7a**,**b** and **8a** in modulating the activity of A_{2B} AR agonists were determined by assessing the effects of different compound concentrations (ranging from 1 nM to 10 μM), on cAMP accumulation induced by an EC_{50} concentration of the agonist NECA (100 nM). The resulting concentration–response curves (Fig. 1A) indicated that **7a**,**b** and **8a** exhibit similar submicromolar potencies at A_{2B} AR, with EC_{50} values between 250 nM and 446 nM.

Compound **7b**, selected as representative of the three compounds, was also found to potentiate the cAMP accumulation induced by the selective A_{2B} AR agonist BAY 60-6583 as well as by adenosine with EC_{50} values of 2390 ± 660 nM and 2550 ± 350 nM, respectively (Fig. 1B).

Subsequently, the effects of **7a**,**b** and **8a** on the potency and efficacy of different A_{2B} AR agonists (NECA, BAY 60-6583 and adenosine) were evaluated by assessing agonist concentration–response curves in the absence or presence of three different concentrations of each compound (100 nM, 500 nM, and 10 μM). All compounds enhanced the efficacy of NECA in stimulating cAMP accumulation without affecting agonist potency (Fig. 2). Similarly, **7b** enhanced the efficacy, but not the potency, of BAY 60-6583 and adenosine (Fig. 3). EC_{50} and E_{max} values of A_{2B} AR agonists obtained in the absence or presence of the tested compounds are reported in Table 2. These results suggest that **7a**,**b** and **8a** behave as positive allosteric modulators of A_{2B} AR by potentiating the efficacy of agonists in activating cAMP intracellular pathway.

3.1.2. Dissociation kinetic assays

Allosteric modulators of GPCRs typically alter the dissociation rate of orthosteric ligands from their binding site [46]. To verify whether **7a**,**b** and **8a** truly acted as allosteric modulators of A_{2B} AR, [^{3}H]NECA dissociation kinetic experiments were performed. In the absence of any of the tested compounds, dissociation of [^{3}H]NECA, induced by an excess of NECA at 25 °C, was completed in 120 min and characterized by a K_{off} value of 0.0162 ± 0.0021 min^{-1} (Fig. 4). When the experiments were performed in the presence of **7b**, selected as representative of the three compounds, a significant reduction in the radioligand dissociation constant was observed (K_{off} value of 0.0086 ± 0.0004 min^{-1}; $p < 0.01$ vs control), demonstrating that the positive modulation of A_{2B} AR exerted by our compounds is indeed of allosteric type.

3.1.3. Equilibrium binding assays

Then, the effects of **7b** on A_{2B} AR ligand binding to orthosteric site at equilibrium were evaluated. As it is well known that allosteric modulators may exert different effects on agonist vs antagonist binding mode [48], we performed competition binding experiments using different agonists as well as different displaceable radioligands in the presence or absence of the tested compound.

Compound **7b** increased the ability of NECA to displace the selective A_{2B} AR antagonist [^{3}H]MRS 1754 from its binding site without any significant effect on agonist affinity (control: IC_{50} = 298.5 ± 21.2 nM; +**7b**: IC_{50} = 320.4 ± 24.4 nM, $p > 0.05$; Fig. 5A).

When [^{3}H]NECA was employed as the displaceable radioligand and the selective A_{2B} AR agonist BAY 60-6583 as the displacing ligand, the binding inhibition curve best fitted a two-site equation model, suggesting that BAY 60-6583 recognizes two distinct affinity states of this receptor (IC_{50high} = 0.0368 ± 0.0021 nM; IC_{50low} = 83.8 ± 7.9 nM; Fig. 5B).

Compound **7b** induced a significant downward shift of BAY 60-6583 binding inhibition curve without affecting agonist affinity at

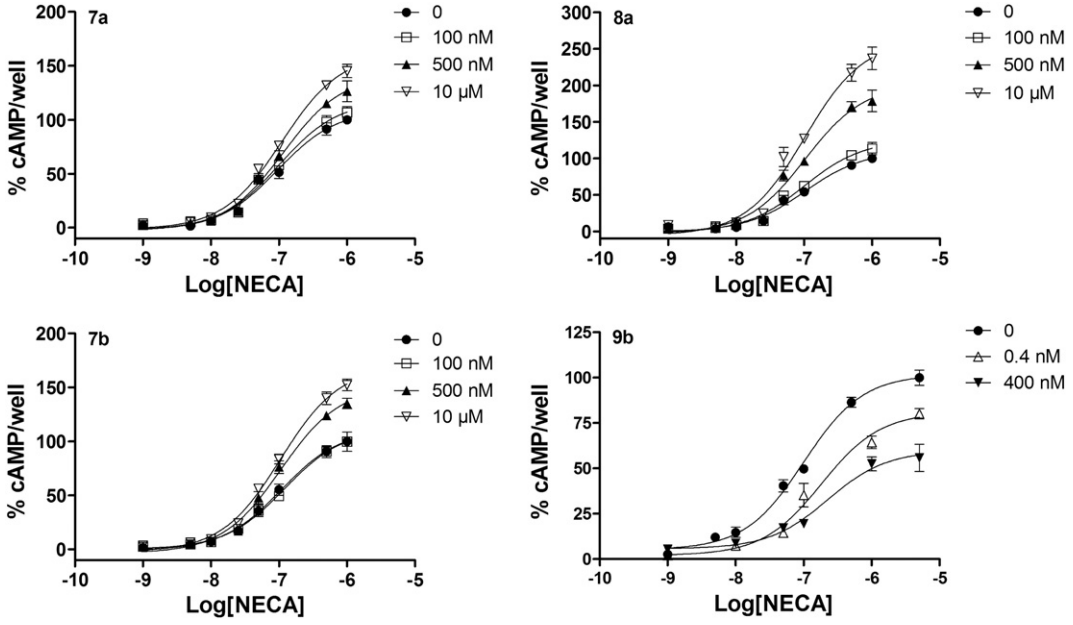

Fig. 2. Modulatory effects of **7a**, **7b**, **8a** and **9b** on the potency and efficacy of the agonist NECA, as measured in a cAMP accumulation assay using intact CHO cells stably expressing human A_{2B} AR. For compounds **7a**, **7b** and **8a** the cells were treated with different concentrations (1 nM–10 μM) of NECA in the absence or presence of three different concentrations (100 nM, 500 nM, 10 μM) of each tested compound. For **9b** the cells were treated with different concentrations of NECA in the absence or presence of two different concentrations (0.4 nM, 400 nM) of the tested compound. After 15 min incubation, the reaction was stopped and the intracellular cAMP levels were quantified. The data are expressed as the percentage of cAMP/well versus the maximal NECA effect, which was set to 100%, and represent the mean ± SEM of at least three different experiments. Each experiment was performed in duplicate.

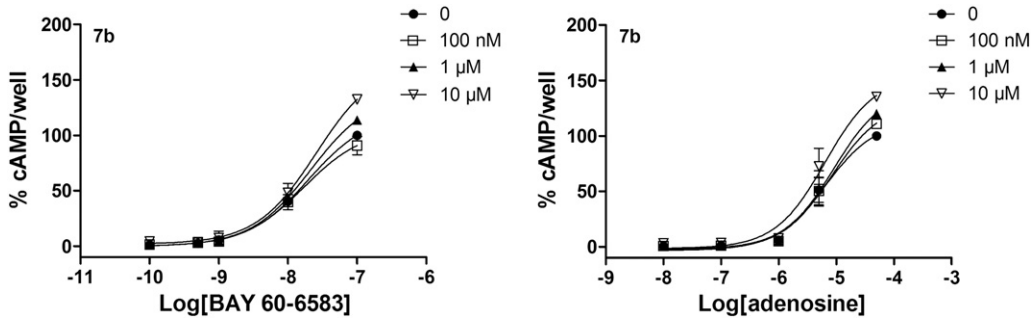

Fig. 3. Effect of **7b** on BAY 60-6583 and adenosine-mediated cAMP accumulation in human A_{2B} AR-transfected CHO cells. CHO cells were treated with different concentrations of BAY 60-6583 or adenosine, in the absence or presence of the indicated concentration of the tested compound. After 15 min incubation, the reaction was stopped and the intracellular cAMP levels were quantified. The data are expressed as the percentage of cAMP/well versus the maximal agonist effect, which was set to 100%, and represent the mean ± SEM of at least three different experiments. Each experiment was performed in duplicate.

both affinity sites of A_{2B} AR (IC_{50high} = 0.0300 ± 0.0021 nM; IC_{50low} = 110.7 ± 18.3 nM; $p > 0.05$ vs control).

These data support the hypothesis that **7b** enhances agonist efficacy by favoring the receptor active state without altering the orthosteric site so as to reduce agonist potency.

3.1.4. [^{35}S]GTPγS binding assays

To deeply investigate the effects of **7b** on the efficiency of A_{2B} AR-G protein coupling, [^{35}S]GTPγS binding experiments were performed. The [^{35}S]GTPγS assay measures the level of G protein activation following occupation of a GPCR by an agonist, by determining the binding of the radiolabeled non-hydrolyzable analog of GTP to the Gα subunit [49].

In control cells, BAY 60-6583 induced a significant stimulation of [^{35}S]GTPγS binding to activated G proteins in a concentration-dependent manner and with a maximal effect of 155.2 ± 4.2%. As shown in Fig. 6, **7b** significantly increased the efficacy of BAY 60-6583 to stimulate guanine nucleotide exchange (1 μM **7b**: E_{max} = 175.0 ± 6.6%, $p < 0.05$; 10 μM **7b**: E_{max} = 198.8 ± 6.9%, $p < 0.001$) without any effect on agonist potency (control: EC_{50} = 4.16 ± 0.46 nM, $p > 0.05$; 1 μM **7b**: EC_{50} = 5.45 ± 0.60 nM, $p > 0.05$; 10 μM **7b**: EC_{50} = 8.25 ± 0.84 nM, $p > 0.05$). It is worth noticing that the upward shifts produced by **7b** on the [^{35}S]GTPγS binding curve shown in Fig. 6 parallel the upward shifts produced by the same compound on the agonist concentration–response curves shown in Figs. 2 and 3.

We also evaluated the effects of **7b** on BAY 60-6583 binding activity in the presence of GTPγS. Compound **7b** was able to completely counteract the detrimental effect of GTPγS on the binding of BAY 60-6583 to A_{2B} AR (Fig. 7), thus confirming its favorable effect on receptor-G protein coupling.

3.2. Pharmacological profile of compounds **8b**,**c** and **9a**,**b** as negative allosteric modulators of A_{2B} AR

3.2.1. cAMP functional assays

As reported in Table 1, all the compounds, when tested alone at 10 μM concentration, did not affect intracellular cAMP levels. In addition, none of these compounds were able to modulate the effects of EC_{50} concentration of the agonist NECA in the A_1, A_{2A}, and A_3 AR-transfected CHO cells, with the exception of **8b**, which acted as A_1 AR antagonist [38]. Compounds **8b**,**c** and **9a**,**b** were able to significantly counteract the NECA-mediated increase of cAMP in CHO cells expressing A_{2B} AR, thus suggesting that these compounds may behave either as selective antagonists or negative modulators of A_{2B} AR (Table 1).

Additional functional assays based on cAMP determination were performed using different concentrations, ranging from 0.01 nM to 10 μM, of these compounds. As shown in Fig. 8, **8b**,**c** and **9a**,**b** counteracted the cAMP accumulation induced by an EC_{50} concentration of the agonist NECA (100 nM) in a concentration–dependent manner. The concentration–response curves of **8b** and **9a**,**b** best fitted a two-site equation model, suggesting that these compounds recognize two sites of A_{2B} AR with different affinities. The IC_{50} values obtained for the high (H) and low (L) affinity states are reported inside the graphs and are in the subnanomolar and micromolar range, respectively. Conversely, the concentration–response curve

Table 2
Effects of compounds **7a**, **7b**, **8a**, and **9b** on the efficacy and potency of A_{2B} AR agonists determined in a cAMP functional assay using CHO cells stably expressing the human A_{2B} AR.

	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
	NECA		Adenosine		BAY 60-6583	
Alone	101.6 ± 3.5	100	4200 ± 120	100	16.6 ± 14	100
+**7a** 100 nM	99.3 ± 3.6	106.8 ± 5.2				
500 nM	116.2 ± 7.4	126.4 ± 9.6				
10 μM	110.4 ± 8.3	145.0 ± 6.2**				
+**7b** 100 nM	120.1 ± 9.3	99.8 ± 9.1	5800 ± 700	110.8 ± 0.5*	15.4 ± 6.5	90.7 ± 8.2
500 nM	106.4 ± 7.4	135.1 ± 4.8**				
1.0 μM	–	–	5800 ± 1.2	116.8 ± 3.4**	18.5 ± 3.9	113.8 ± 2.8*
10 μM	105.6 ± 9.3	152.3 ± 5.5***	4500 ± 600	135.2 ± 1.5***	20.4 ± 6.5	132.4 ± 3.9**
+**8a** 100 nM	99.1 ± 5.0	113.4 ± 8.2				
500 nM	104.1 ± 4.2	178.5 ± 14.8**				
10 μM	102.4 ± 6.1	236.8 ± 15.4***				
Alone	99.6 ± 16.0	100				
+**9b** 0.4 nM	126.2 ± 10.4	80.2 ± 2.8*				
400 nM	132.0 ± 23.1*	55.8 ± 7.5**				

* $p < 0.05$ vs agonist alone.
** $p < 0.01$ vs agonist alone.
*** $p < 0.001$ vs agonist alone.

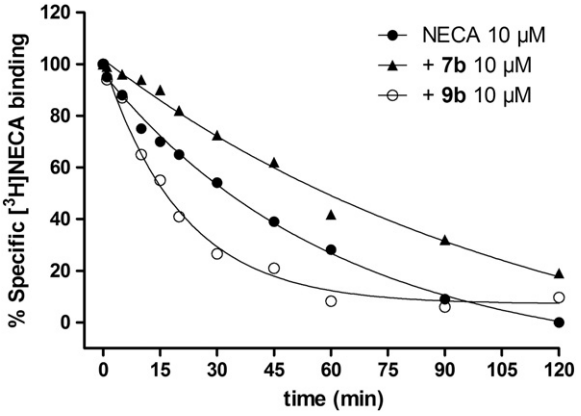

Fig. 4. Effects of **7b** and **9b** on dissociation kinetics of [^{3}H]NECA from human A_{2B} AR cell membranes. [^{3}H]NECA (30 nM) was incubated with 25 μg CHO cell membranes for 180 min at 25 °C. Dissociation was initiated by the addition of 10 μM NECA in the absence or presence of 10 μM of the tested compound (**7b** or **9b**). Samples were filtered after incubation at the indicated time points. The data shown were derived from one experiment performed in duplicate and are typical of the results obtained in three independent experiments.

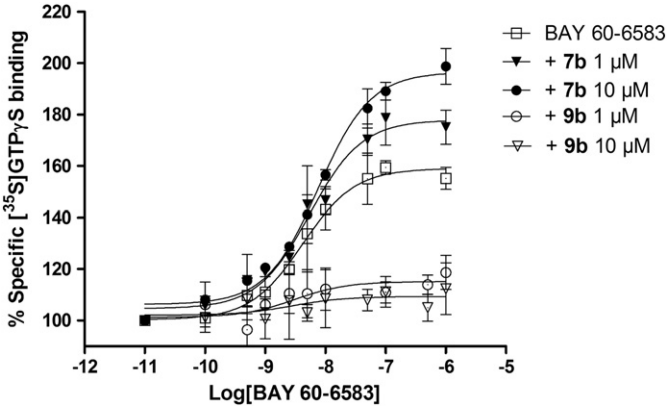

Fig. 6. Effects of **7b** and **9b** on A_{2B} AR-G protein coupling: [^{35}S]GTPγS binding assay. Cell membranes were treated with different concentrations of BAY 60-6583 (0.01 nM–1 μM), in the absence or presence of different concentrations (1 μM–10 μM) of the tested compound (**7b** or **9b**) and then incubated with 0.2 nM [^{35}S]GTPγS. The experimental procedures are reported in the Experimental section. Data are expressed as percentage of [^{35}S]GTPγS specific binding over basal value (set to 100%) and represent the mean ± SEM of three different experiments. Each experiment was performed in duplicate.

of **8c** fitted a one site-equation model, suggesting that this compound recognizes a unique site of A_{2B} AR. The IC_{50} value obtained for **8c** is reported inside the graph and is in the low nanomolar range.

To investigate whether **8b**,**c** and **9a**,**b** act as A_{2B} AR orthosteric antagonists or negative allosteric modulators, we performed additional experiments on **9b** selected as representative of these compounds.

We first evaluated the effect of **9b** on the NECA concentration–response curve using two concentrations of the tested compound corresponding to the IC_{50} values of the high and low affinity sites of A_{2B} AR recognized by this compound. In such an experiment, **9b** induced a downward as well as a right shift of the NECA concentration–response curve meaning that this compound reduces either efficacy and potency of the agonist (Table 2 and Fig. 2), respectively, likely acting as a negative allosteric modulator of A_{2B} AR.

3.2.2. Dissociation kinetic assays

To unambiguously establish whether the negative modulation of A_{2B} AR exerted by **8b**,**c** and **9a**,**b** was based on an allosteric mechanism, we analyzed the effects of **9b**, selected as representative of the four compounds, on [^{3}H]NECA dissociation kinetic. Compound **9b** significantly increased the dissociation rate of [^{3}H]NECA from A_{2B} AR determined by an excess of NECA, consistently with a typical profile of negative allosteric modulator (K_{off} = 0.0481 ± 0.0026, $p < 0.001$ vs control; Fig. 4).

3.2.3. Equilibrium binding assays

In competition binding experiments, **9b** produced a significant upward and right shift of BAY 60-6583 inhibition curve towards the low affinity binding state (BAY 60-6583: IC_{50high} = 0.0368 ± 0.0021 nM; IC_{50low} = 83.8 ± 7.9 nM; BAY 60-6583 + **9b**: IC_{50} = 2.22 ± 0.15 nM; Fig. 5B). This suggests that **9b** may alter the conformational equilibrium of A_{2B} AR in favor of the resting states and, at the same time, decreases the affinity of the active states for agonist BAY 60-6583.

3.2.4. [^{35}S]GTPγS binding assays

To evaluate the effects of **9b** on A_{2B} AR-G protein coupling, [^{35}S]GTPγS binding experiments were carried out similarly to those previously performed on **7b**. Compound **9b**, used at two different concentrations (1 and 10 μM), caused a significant impairment in the agonist-mediated stimulation of guanine nucleotide exchange (Fig. 6). These results suggest that **9b** is able to decrease the efficacy of A_{2B} AR agonists by interfering with receptor-G protein coupling and, consequently, with the agonist functional response. It is worth outlining that the concentration-dependent downward shifts produced by **9b** on the [^{35}S]GTPγS binding curve shown in Fig. 6 parallel the right downward shifts produced by the same compound on the agonist concentration–response curves shown in Fig. 2.

Furthermore, the data depicted in Fig. 7 demonstrated that **9b** counteracted the binding of BAY 60-6583 to A_{2B} AR to an extent similar

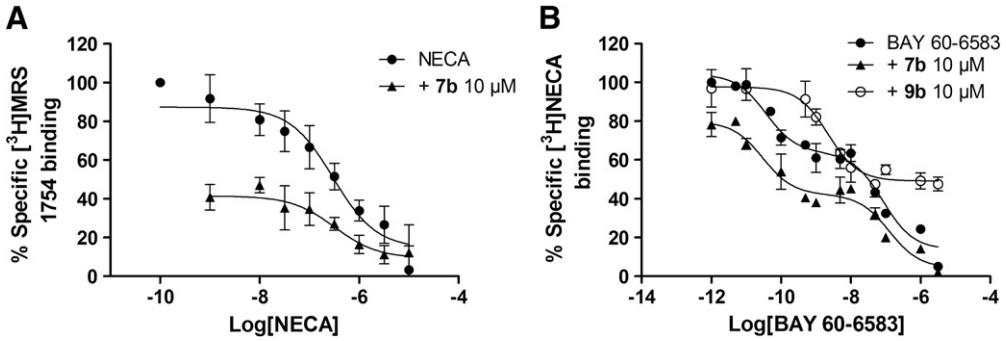

Fig. 5. Effects of **7b** and **9b** on the binding of the non-selective agonist NECA and the selective agonist BAY 60-6583 to A_{2B} AR. (A) Cell membranes (50 μg proteins) were incubated with 0.7 nM [^{3}H]MRS 1754 and different concentrations of NECA in the absence or presence of 10 μM **7b**. (B) Cell membranes (25 μg proteins) were incubated with 30 nM [^{3}H]NECA and different concentrations of BAY 60-6583, in the absence or presence of 10 μM of the tested compound (**7b** or **9b**). After reaching equilibrium, samples were filtered and the bound radioactivity was counted. Data are expressed as percentage of specific binding versus basal value (set to 100%) and represent the mean ± SEM of three different experiments.

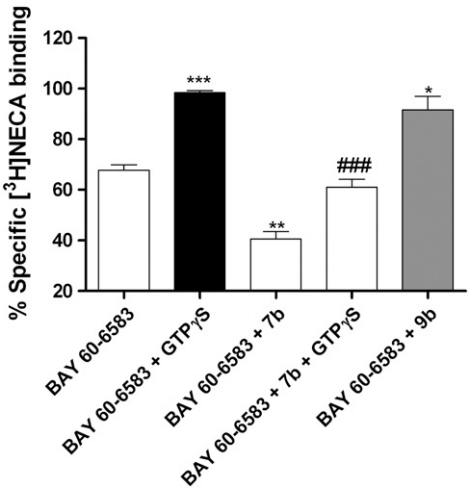

Fig. 7. Effects of **7b** on GTPγS mediated A_{2B} AR-G protein uncoupling. Cell membranes (25 μg proteins) were incubated with 30 nM [^{3}H]NECA, 0.5 nM BAY 60-6583 and 10 μM of the tested compound in the absence or presence of 10 μM GTPγS. After 180 min incubation, samples were filtered and the bound radioactivity was counted. Effects of **9b** on the displacement of [^{3}H]NECA from BAY 60-6583 were obtained in similar conditions in the absence of GTPγS. Data are expressed as percentage of [^{3}H]NECA specific binding over basal value (set to 100%) and represent the mean ± SEM of three different experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs BAY 60-6583; $^{###}p < 0.001$ vs BAY 60-6583.

to that exhibited by GTPγS, suggesting that this compound favors the receptor uncoupled state with an efficacy comparable to that of GTPγS. It is possible that **9b** might exert its detrimental effect on A_{2B} AR-G protein coupling by accelerating the conformational switching between the resting and the active states of this receptor so as to put out of phase the recognition and coupling processes.

4. Conclusions

We have described the pharmacological characterization of the 1-benzyl-3-ketoindoles **7**–**9** as positive or negative allosteric modulators of human A_{2B} AR.

In our experiments, **7a**,**b** and **8a** acted as positive allosteric modulators of A_{2B} AR by enhancing efficacy, but not potency, of agonists. The allosteric interaction of these compounds with A_{2B} AR could be more easily discerned by combining experiments performed under either equilibrium and, especially, non-equilibrium conditions. To this regard, it has been demonstrated for different GPCRs that an allosteric modulator may promote specific receptor conformations that are not evident in radioligand equilibrium binding assays [46]. In addition, several studies report that the effects of allosteric modulators on agonist efficacy and potency are not necessarily related properties [50–53]. Therefore, it is plausible that **7a**,**b** and **8a** behave as positive allosteric modulators of A_{2B} AR by affecting specific conformational states of this receptor that are not related with agonist potency, but rather to functional coupling to the intracellular signaling system.

The experiments performed on **8b**,**c** and **9a**,**b** suggested that these compounds behave as negative allosteric modulators of A_{2B} AR by decreasing both efficacy and potency of agonists. The above compounds probably interact with A_{2B} AR by shifting its conformational states toward the resting ones, and by changing the conformation of the orthosteric site so as to reduce agonist potency.

Because of the indirect mechanism of receptor modulation, allosteric modulators of GPCRs offer several pharmacological advantages compared to agonists and antagonists. Particularly, they tune tissue responses only when and where the endogenous agonist is present to exert its patho-physiological effects; moreover, they produce effects that are generally limited in time and place, being dependent on the release of the endogenous agonist [25–28]. The 1-benzyl-3-ketoindoles **7**–**9**, acting as positive or negative allosteric modulators of A_{2B} AR, may represent pharmacological tools useful for the development of

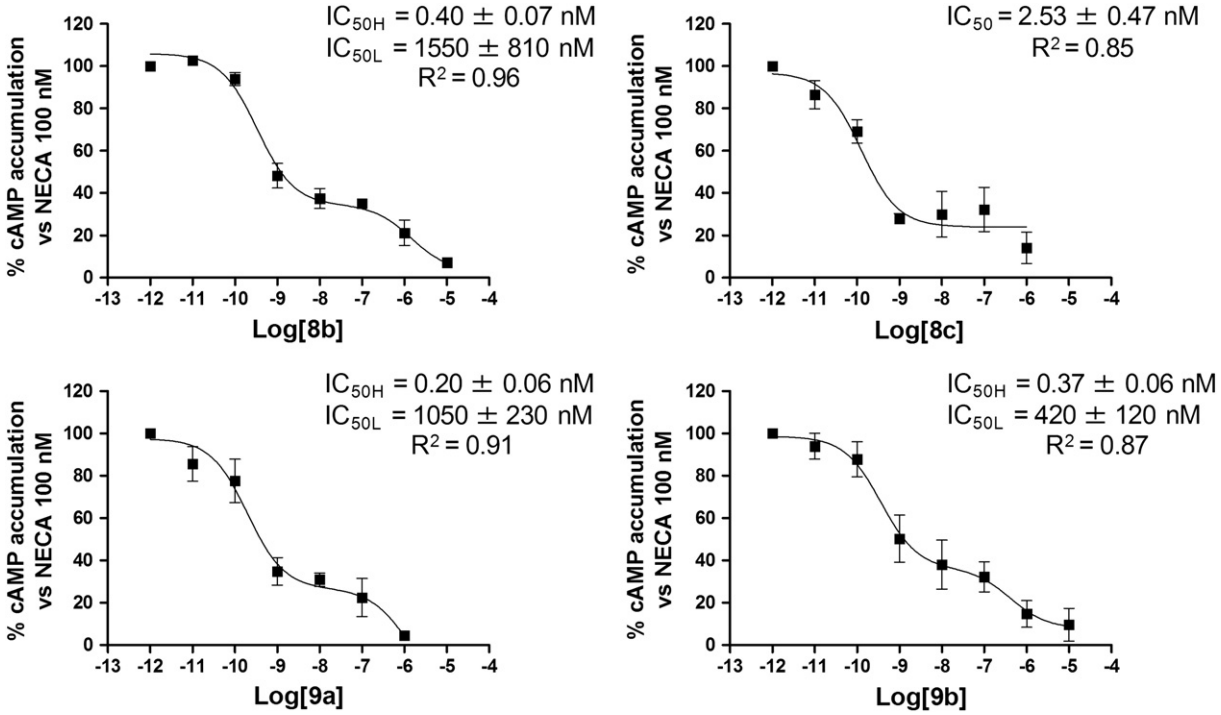

Fig. 8. Effects of **8b**, **8c**, **9a** and **9b** on NECA-mediated cAMP accumulation in human A_{2B} AR-transfected CHO cells. CHO cells were treated with EC_{50} NECA concentration (100 nM) in the absence or presence of different concentrations (0.001 nM–10 μM) of the tested compound. After 15 min incubation, the reaction was stopped and the intracellular cAMP levels were quantified. The results are expressed as the percentage of NECA-stimulated cAMP levels, which was set to 100%. The data represent the mean ± SEM of at least three different experiments. Each experiment was performed in duplicate.

new therapeutic agents to treat pathologies related to abnormal stimulation of this receptor. Allosteric modulators of A_{2B} AR may offer several clinical advantages over agonists and antagonists of this receptor in terms of selectivity of action thanks to the phenomenon of pharmacological cooperativity. A_{2B} AR allosteric modulators should display a non-detectable neutral cooperativity with adenosine at A_1, A_{2A} and A_3 AR subtypes, whereas they should exhibit a positive or a negative cooperativity at the A_{2B} subtype leading to a significant tissue selectivity and a resulting enlargement of the therapeutic window. Given the role played by A_{2B} AR in several physiological and pathological processes, including glucose metabolism, angiogenesis induction, growth of some tumors, intestinal inflammation, myocardial ischemia, acute lung and kidney injury, the positive and negative allosteric modulators of this receptor represent promising tools for future research work in this field.

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