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Preliminary communication

Modulation of A_{2B} adenosine receptor by 1-Benzyl-3-ketoindole derivatives

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ABSTRACT

We have disclosed a series of 1-benzyl-3-ketoindole derivatives acting as either positive or negative modulators of the human A_{2B} adenosine receptor (A_{2B} AR) depending on small differences in their side chain. The new compounds were designed taking into account structural similarities between AR antagonists and ligands of the $GABA_A$/benzodiazepine receptor. All compounds resulted totally inactive at A_{2A} and A_3 ARs and showed small (**8a**,**b**) or none (**7a**,**b**, **8c** and **9a**,**b**) affinity for A_1 AR. When tested on A_{2B} AR-transfected CHO cells, **7a**,**b** and **8a** acted as positive modulators, whereas **8b**,**c** and **9a**,**b** acted as negative modulators, enhancing or weakening the NECA-induced increase of cAMP levels, respectively. Compounds **7**–**9** might be regarded as useful biological and pharmacological tools to explore the therapeutic potential of A_{2B} AR modulators, while their 3-ketoindole scaffold might be taken as a reference to design new analogs.

1. Introduction

Adenosine is an endogenous purine nucleoside that modulates a variety of physiological processes by triggering specific cell membrane G-protein-coupled receptors (GPCRs) known as adenosine receptors (ARs). ARs are widely distributed in mammalian tissues and have been classified into four subclasses: A_1, A_{2A}, A_{2B}, and A_3 [1–3]. A_{2B} AR is defined as the "low-affinity" subtype because requires high micromolar concentrations of adenosine to be activated [4–6]. It couples to Gs proteins, thus stimulating adenylate cyclase and cAMP accumulation, as well as Gq proteins, resulting in phospholipase C activation and enhancement of the inositol trisphosphate and diacylglycerol pathways [7]. A_{2B} AR regulates a number of physiological and pathological events that involve lungs, mast cells, eyes, the gastrointestinal tract, bladder, adipose tissue, brain, kidneys, liver, and other tissues [2,6]. This receptor is the least well-characterized among the ARs primarily due to the lack of suitable, specific ligands [3,8–10]. Recently, several potent and selective A_{2B} AR agonists have been identified, and a phenylpyridinesulfanyl acetamide derivative (BAY 60-6583, **1** in Chart 1), is currently in preclinical studies for the treatment of atherosclerosis and coronary artery disorders [10,11]. To the best of our knowledge, no allosteric modulators of A_{2B} AR have been described in the literature thus far [12–14]. Because of the involvement of A_{2B} AR in several physiological and pathological processes, including glucose metabolism [15], angiogenesis induction [16,17], the growth and development of some tumors [18], and inflammation [19,20], potent and selective A_{2B} AR antagonists are currently being developed as candidates for the treatment of diabetic retinopathy and cancer [21,22], colitis [23,24], and asthma [25–27]. Several classes of A_{2B} AR antagonists, including compounds **2**–**6** represented in Chart 1, have been described to date [3,8–10,26]: pyrrolopyrimidines (**2**) [28], pyrazolotriazolopyrimidines (**3**) [29], 2-aminopyrazines (**4**) [30], xanthines (**5**) [31], and triazinobenzimidazolones (**6**) [32].

Compound **6** was recently identified by a screening study of our "in house" collection of triazinobenzimidazolones, which were originally described as ligands of the $GABA_A$/benzodiazepine receptor (BzR) [33] and subsequently modified to obtain A_1 AR

Abbreviations: ADA, adenosine deaminase; AR, adenosine receptor; cAMP, 3′,5′-cyclic adenosine monophosphate; CHO, Chinese hamster ovary; Cl-IBMECA, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide; DMAP, 4-dimethylaminopyridine; DMEM, Dulbecco's Modified Eagle Medium; GPCRs, G-protein coupled receptors; [^{3}H]DPCPX, [^{3}H]8-cyclopentyl-1,3-dipropylxanthine; [^{3}H]NECA, [^{3}H]5′-*N*-ethylcarboxamideadenosine; [^{125}I]AB-MECA, [^{125}I]4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine; SEM, standard error of mean.

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antagonists [34,35]. This strategy of designing AR antagonists from BzR ligands by simple structural modifications dates back to the discovery of the first non-xanthine AR antagonists [36]. Adopting this approach, we have recently used the indol-3-ylglyoxylamide **7**, which is the prototype of several indole derivatives that we previously reported to be BzR ligands [37–44], as a reference structure to design the following 3-ketoindoles as potential AR antagonists: the *N*-(indol-3-ylglyoxyl)amides **7a**,**b**, the 3-(arylglyoxylyl)indoles **8a**–**c** and the indol-3-ylcarboxamides **9a**,**b** (Chart 2).

These compounds can be regarded as open chain analogs of the triazinobenzimidazolone **6**. Moreover, each compound contains two structural features that are common to the majority of A_{2B} AR antagonists reported in Chart 1: a) three lipophilic moieties connected to a heterocyclic core scaffold, the fused benzene ring, the benzyl attached to the indole nitrogen and the side chain aryl ring, and b) a hydrophilic moiety capable of making hydrogen bonds, the COCONH, COCO and CONH fragments. These two features were hypothesized to be critical for the binding of compound **6** and its derivatives to A_{2B} AR because docking studies suggested that they are involved in hydrophobic contacts and in a hydrogen bond between the ligand carbonyl oxygen and the Asn-254 side chain of the receptor, respectively [32]. This last interaction was reported to be necessary for the affinity of A_{2B} AR antagonists based on X-ray crystallography [45] and mutagenesis data [46].

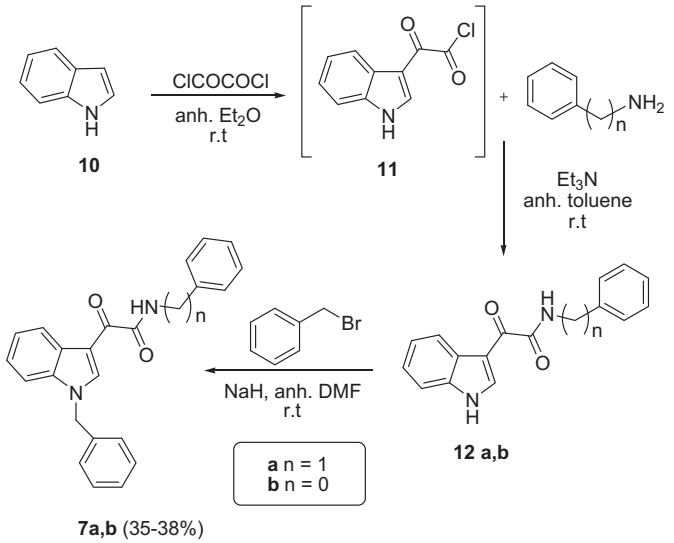
Here, we describe the synthesis and biological evaluation of the 3-ketoindoles **7**–**9** (Chart 2) on human A_1, A_{2A}, A_{2B} and A_3 ARs, which unexpectedly led to the identification of three compounds (**7a**,**b** and **8a**) as positive modulators and four compounds (**8b**,**c** and **9a**,**b**) as negative modulators of A_{2B} AR.

2. Chemistry

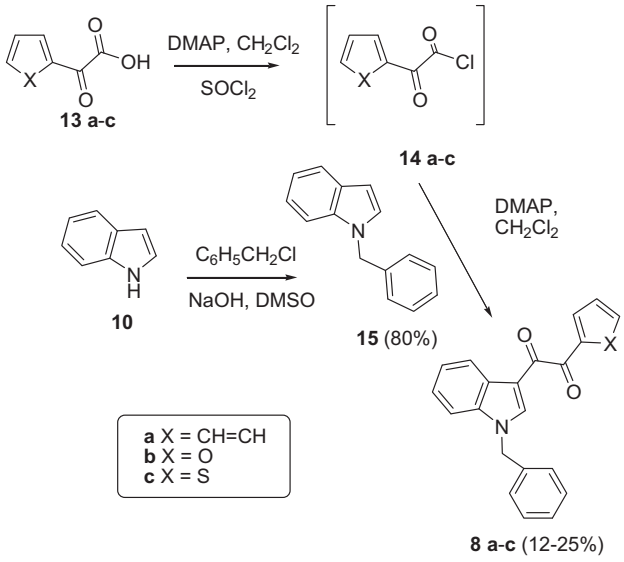
The general procedure employed to prepare compounds **7a**,**b** involved the acylation of commercially available indole **10** with oxalyl chloride to give the corresponding indol-3-ylglyoxyl chloride **11**, which was directly allowed to react with the appropriate amine in the presence of triethylamine in dry toluene solution, to obtain the amides **12a**,**b** (Scheme 1) [37]. Treatment of **12a**,**b** with sodium hydride and subsequent addition of benzyl bromide in dry DMF yielded the target derivatives **7a**,**b**.

The synthesis of compounds **8a**–**c** was achieved by the key intermediate **15**, as shown in Scheme 2. The suitable α-oxoacid (**13a**–**c**) reacted with $SOCl_2$ in the presence of DMAP in dichloromethane to provide the arylchloride (**14a**–**c**) that successively was treated with **15** using DMAP as a base to yield the desired α-diketo derivative **8a**–**c**.

Compounds **9a**,**b** are commercially available (Bionet).



Scheme 1. Synthesis of compounds **7a**,**b**.



Scheme 2. Synthesis of compounds **8a**–**c**.

3. Biological assays

The affinities of compounds **7**–**9** for human A_1, A_{2A} and A_3 ARs were evaluated by competition experiments assessing their respective abilities to displace [^{3}H]DPCPX, [^{3}H]NECA, or [^{125}I]AB-MECA binding from transfected CHO cells [35].

The functional activity of each compound at human A_1, and A_{2B} ARs was evaluated by cAMP assay, essentially following procedures previously described [32].

4. Results and discussion

The binding affinities of the 3-ketoindole derivatives **7**–**9** for human A_1, A_{2A} and A_3 ARs are summarized in Table 1, along with those of DPCPX, NECA, and Cl-IBMECA, which are used as the reference standards.

Table 1
Binding affinity of compounds **7**–**9** to human A_1, A_{2A}, and A_3 ARs.[a]

cpd	A_1 K_i (nM)[b]	A_{2A} K_i (nM)[c]	A_3 K_i (nM)[d]
7a	>10,000	>10,000	>1000
7b	>10,000	>10,000	>1000
8a	161.5 ± 17.4	>10,000	>1000
8b	343.0 ± 15.0	>10,000	>1000
8c	>10,000	>10,000	>1000
9a	>10,000	>10,000	>1000
9b	>10,000	>10,000	>1000
DPCPX	0.50 ± 0.03	337 ± 28	>1000
NECA	14 ± 4	16 ± 3	73 ± 5
Cl-IBMECA	890 ± 61	401 ± 25	0.22 ± 0.02

[a] Data are expressed as means ± SEM derived from an iterative curve-fitting procedure (Prism program, GraphPad, San Diego, CA); percentages refer to extent of inhibition of specific radioligand binding at 10 μM compound concentration.
[b] Displacement of specific [^{3}H]DPCPX binding in membranes obtained from human A_1 AR stably expressed in CHO cells.
[c] Displacement of specific [^{3}H]NECA binding in membranes obtained from human A_{2A} AR stably expressed in CHO cells.
[d] Displacement of specific [^{125}I]AB-MECA binding in membranes obtained from human A3 AR stably expressed in CHO cells.

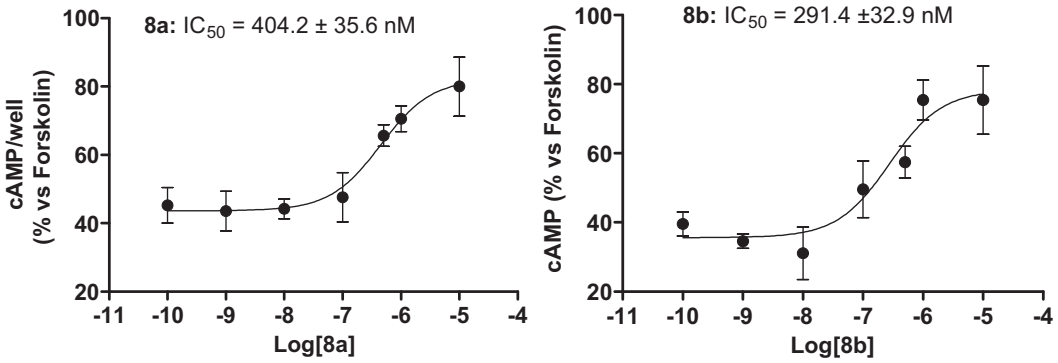


Fig. 1. Effects of **8a** and **8b** on NECA-mediated inhibition of cAMP accumulation in human A_1 AR-transfected CHO cells. CHO cells were treated with 1 μM forskolin and 10 nM NECA in the absence or presence of different concentrations of the compounds (1 nM–10 μM). After 15 min incubation, the reaction was stopped and the intracellular cAMP levels were quantified. The data are expressed as the percentage of the cAMP intracellular levels with respect to forskolin, which was set to 100%, and represent the mean ± SEM of at least three different experiments. Each experiment was performed in duplicate.

None of the compounds displayed any significant binding affinity for A_1, A_{2A} and A_3 ARs, with the exception of **8a** and **8b**. These compounds displayed moderate affinity for A_1 AR, with K_i values in the submicromolar range (**8a** K_i 161.5 nM; **8b** K_i 343.0 nM). In addition, as demonstrated by the functional cAMP assay (Fig. 1), these two compounds were able to counteract the agonist-mediated inhibition of cAMP accumulation in human A_1 AR-transfected CHO cells in a concentration-dependent manner with IC_{50} values that were comparable to the K_i values obtained in the binding assay (**8a** IC_{50} 404.2 nM; **8b** IC_{50} 291.4 nM). These data demonstrate that **8a** and **8b** possess antagonist activities towards A_1 AR.

Compounds **7**–**9** were then evaluated for their ability to modulate the increase in cAMP levels either alone or in the presence of an EC_{50} concentration of the agonist NECA in human A_{2B} AR-transfected CHO cells (Table 2).

When tested alone at a 10 μM concentration, all the compounds did not significantly increase the cAMP levels, demonstrating a lack of A_{2B} AR agonist activity (Table 2).

In the presence of the agonist NECA, **7a**,**b** and **8a** potentiated the effects of NECA, suggesting that these compounds may interact with this receptor as positive modulators.

On the contrary, compounds **8b**,**c** and **9a**,**b** were able to potently counteract the NECA-mediated increase in cAMP in human A_{2B} AR-transfected CHO cells, suggesting that they may act as negative modulators of A_{2B} AR (Table 2).

Interestingly, in the experiments performed on wild-type CHO cells that did not express ARs, compounds **7**–**9** did not show any functional effects (data not shown), which indicates that these compounds do not exert their effects by modulating proteins other than A_{2B} AR.

Taken together, the results of the data discussed thus far allow us to outline only rough structure–efficacy relationships for the interaction of the 3-ketoindoles **7**–**9** with A_{2B} AR. In this series of compounds, **7** and **9** act as either negative or positive modulators depending on the presence of a glyoxylamide or a carboxamide linker, respectively, regardless of the side chain lipophilic moiety, which is a benzyl for **7a** and **9a** and a phenyl for **7b** and **9b**. By contrast, compounds **8** feature a diketo linker, and the ligand–protein interaction depends on the nature of the pendant aromatic ring, with either positive or negative modulation for the phenyl- (**8a**) or 2-furyl/2-thienyl- (**8b**,**c**) substituted compounds, respectively.

5. Conclusions

We have disclosed the 1-benzyl-3-ketoindole substructure as a new scaffold for the obtainment of potent and selective modulators of the A_{2B} AR subtype. Subtle differences in the decoration of this scaffold determined a deep difference in the efficacy of the compounds, permitting to obtain both positive (**7a**,**b** and **8a**) and negative (**8b**,**c** and **9a**,**b**) modulators. Additional experiments are currently being performed in our laboratories to better characterize the pharmacological profile of these compounds and hopefully gain some insights into their mode of interaction with A_{2B} AR. The results of these on-going researches will be published in due course. For the moment being, we can confidently state that our preliminary data indicate that 3-ketoindoles **7**–**9** are useful tools for the development of novel A_{2B} AR modulators, thus paving the way to explore their therapeutic potential.

6. Experimental

6.1. Chemistry

Melting points were determined using a Büchi apparatus B 540 and are uncorrected. Routine nuclear magnetic resonance spectra were recorded on a Varian Mercuryplus$_{400}$ spectrometer operating at 400 MHz for ^{1}H nucleus and 100 MHz for ^{13}C nucleus, respectively. Evaporation was performed in vacuo (rotary evaporator). Analytical TLC was carried out on Merck 0.2 mm precoated silica gel aluminum sheets (60 F-254). Silica gel 60 (230–400 mesh) was used for column flash-chromatography. Combustion analyses were used to determine the purity of target compounds. All compounds showed ≥95% purity. The *N*-benzyl-indol-3-ylglyoxylamide **12a** and *N*-phenyl-indol-3-ylglyoxylamide **12b** were prepared according to a reported procedure [37]. Compounds **9a**,**b** are commercially available (Bionet); their structure were confirmed by ^{1}H NMR and elemental analyses.

6.1.1. Synthesis of N-benzyl- or N-phenyl-1-benzyl-indol-3-ylglyoxylamides *7a*,*b*

Sodium hydride (0.11 mmol, 60% dispersion in mineral oil) was portion wise added to an ice-cooled solution of **12a**,**b** (10 mmol) in 10 mL of dry DMF, and the mixture was stirred at room temperature for 1 h. Benzyl bromide (1.4 mL, 10 mmol) was added, and stirring was continued for 24 h. The reaction mixture was diluted with water and ice and the formed precipitate was collected by filtration and purified by flash chromatography (eluting system: petroleum ether (60–80 °C) and ethyl acetate in ratio 7:3).

*6.1.1.1. N-Benzyl-2-(1-benzyl-1H-indol-3-yl)-2-oxoacetamide (**7a**).* Yield: 45%, mp 125.1–127.0 °C (toluene). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 9.31 (bs exch., 1H, NH–CH_2); 8.96 (s, 1H, H-2); 8.25–8.23 (m, 1H, H-4); 7.58–7.56 (m, 1H, Ar–H); 7.31–7.26 (m, 12H, Ar–H); 5.57 (s, 2H, 1-CH_2Ph); 4.40 (d, 2H, J = 3.2 Hz, NHCH_2Ph). ^{13}C

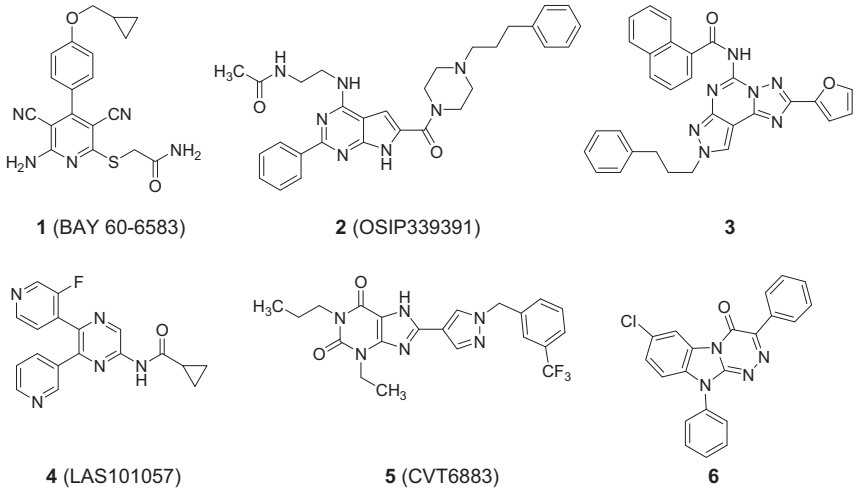


Chart 1. The structures of the compounds reported in the literature to be A_{2B} AR agonists (**1**) and antagonists (**2**–**6**).

NMR (100 MHz, DMSO-d_6, δ ppm): 181.1; 162.8; 140.5; 138.3; 136.0; 135.7; 128.1; 127.7; 127.2; 127.0; 126.7; 126.3; 126.2; 123.0; 122.3; 121.0; 111.0; 110.9; 49.3; 41.6. Anal. Calcd. for $C_{24}H_{20}N_2O_2$ (%): C, 78.24; H, 5.47; N, 7.60. Found: C, 78.29; H, 5.51; N, 7.64.

*6.1.1.2. 2-(1-Benzyl-1H-indol-3-yl)-2-oxo-N-phenylacetamide (**7b**).* Yield: 50%, mp 134.8–136.9 °C (toluene). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 10.69 (s exch., 1H, NH); 8.97 (s, 1H, H-2); 8.30–8.27 (m, 1H, H-4); 7.86–7.82 (m, 2H, Ar–H); 7.62–7.59 (m, 1H, Ar–H); 7.40–7.10 (m, 9H, Ar–H); 5.59 (s, 2H, CH_2Ph). ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 181.4; 162.0; 141.0; 137.8; 136.4; 136.2; 128.5; 127.6; 127.2; 126.7; 124.0; 123.5; 122.9; 121.3; 120.1; 111.4; 111.2; 49.8. Anal. Calcd. for $C_{23}H_{18}N_2O_2$ (%): C, 77.95; H, 5.12; N, 7.90. Found: C, 77.99; H, 5.16; N, 7.93.

*6.1.2. Synthesis of 1-(1-benzyl-1H-indol-3-yl)-2-arylethane-1,2-diones **8a**–**c***

Thionyl chloride (0.2 mL; 2.4 mmol) and then a mixture of the proper α-oxoacid (2.4 mmol) in dry CH_2Cl_2 (10 mL) were added drop wise to a cooled (10 °C) solution of DMAP (0.30 g, 2.4 mmol) in the same solvent (10 mL). The mixture was allowed to warm at room temperature and stirred for 3 h. DMAP (0.30 g, 2.4 mmol) and 1-benzyl-1*H*-indole (**15**) (0.50 g, 2.4 mmol) in anhydrous CH_2Cl_2 (3 mL) were added to the cooled (0 °C) mixture. The reaction mixture was kept refluxing for 2 h and added with water (20 mL). The organic layer was separated and the aqueous phase was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic phases were washed with an aqueous saturated solution of NaCl (15 mL), dried (Na_2SO_4) and evaporated to dryness. The crude product was purified by chromatography to give the desired products.

*6.1.2.1. 1-(1-Benzyl-1H-indol-3-yl)-2-phenylethane-1,2-dione (**8a**).* Ethyl acetate:petroleum ether 1:3 v/v as eluent. Yield 21%, mp 102.1–103.0 °C (EtOH). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.48 (s, 1H, H-2); 8.24 (d, 1H, J = 7.3 Hz, H-4); 7.99 (d, 2H, H–Ar); 7.76 (t, 1H, H–Ar); 7.63–7.58 (m, 3H, H-7 and H–Ar); 7.34–7.25 (m, 8H, H-5, H-6, H–Ar); 5.54 (s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 193.8; 188.1; 140.6; 136.8; 136.5; 134.8; 132.8; 129.8; 129.2; 128.7; 127.8; 127.3; 125.7; 124.0; 123.3; 121.5; 111.9; 111.8; 49.9. Anal. Calcd. for $C_{23}H_{17}NO_2$ (%): C, 81.40; H, 5.05; N, 4.13. Found: C, 81.55; H, 5.28; N, 4.01.

*6.1.2.2. 1-(1-Benzyl-1H-indol-3-yl)-2-(furan-2-yl)ethane-1,2-dione (**8b**).* Ethyl acetate:petroleum ether 1:3 v/v as eluent. Yield 34%, mp 132.9–133.5 °C (cyclohexane). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.58 (s, 1H, H-2); 8.24 (pd, 1H, H-4); 8.21 (d, 1H, J = 1.5 Hz,

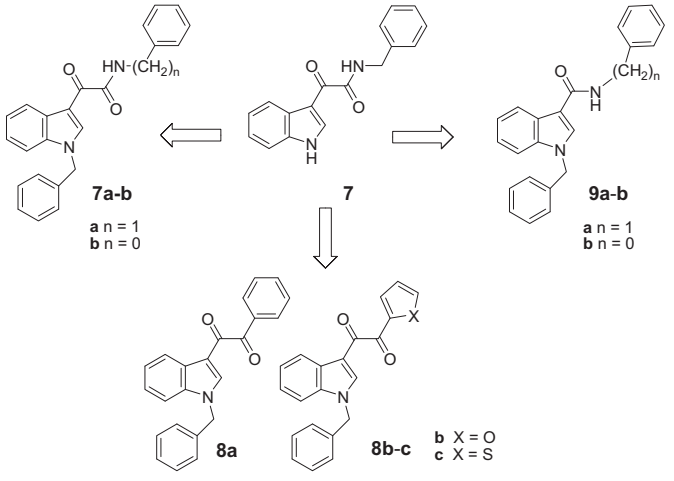


Chart 2. The structures of the new 3-ketoindole derivatives **7a**,**b**, **8a**–**c**, **9a**,**b** designed taking compound **7**, a ligand of the benzodiazepine receptor, as a reference.

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

cpd	% cAMP production (vs agonist maximal effect, 100%)[b]	
	Alone	+ NECA
7a	5.2 ± 1.0	75.9 ± 0.4*
7b	6.2 ± 1.3	83.6 ± 4.4*
8a	8.3 ± 1.2	127.0 ± 5.7***
8b	9.2 ± 1.2	1.0 ± 0.9
8c	8.4 ± 1.3	2.5 ± 1.1
9a	8.1 ± 1.2	1.2 ± 1.0
9b	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_{2B} ARs (see Biological section). Each compound was tested alone or in the presence of an EC_{50} concentration of agonist NECA (determined on the same day as each assay).

[b] Data are expressed as percentage of cAMP production vs agonist maximal effect (100%). All data represent the mean ± SEM of at least three different experiments each performed in duplicate. *$P < 0.05$, ***$P < 0.001$ vs agonist alone.

H-3′); 7.62–7.58 (m, 2H, H-7 and H-5′); 7.38–7.23 (m, 7H, H-5, H-6, H–Ar); 6.82 (m, 1H, H-4′); 5.56 (s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 185.3; 180.3; 150.2; 149.4; 140.8; 136.7; 136.5; 128.7; 127.8; 127.3; 126.1; 123.9; 123.8; 123.2; 121.5; 113.3; 111.8; 111.2; 49.9. Anal. Calcd. for $C_{21}H_{15}NO_3$ (%): C, 76.58; H, 4.59; N, 4.25. Found: C, 77.29; H, 4.81; N, 4.07.

*6.1.2.3. 1-(1-Benzyl-1H-indol-3-yl)-2-(thiophen-2-yl)ethane-1,2-dione (**8c**).* Ethyl acetate:petroleum ether 1:2 v/v as eluent. Yield 12%, mp 102.3 °C dec. (*n*-hexane). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.61 (s, 1H, H-2); 8.26 (d, 1H, H-4); 8.23 (d, 1H, J = 1.5 Hz, H-5′); 7.99 (d, 1H, H-3′); 7.61 (d, 1H, H-7); 7.36–7.25 (m, 8H, H-5, H-6, H-4′, H–Ar); 5.57 (s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 185.3; 185.0; 141.0; 138.8; 138.0; 137.0; 136.7; 136.5; 129.2; 128.7; 127.8; 127.3; 126.2; 124.0; 123.3; 121.6; 111.8; 111.2; 49.9. Anal. Calcd. for $C_{21}H_{15}NO_2S$ (%): C, 73.02; H, 4.38; N, 4.06. Found: C, 74.12; H, 4.61; N, 3.91.

*6.1.3. ^{1}H NMR and elemental analyses of compounds **9a**,**b***

*6.1.3.1. N,1-Dibenzyl-1H-indole-3-carboxamide (**9a**).* ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.50 (t exch., 1H, J = 5.7 Hz, NH–CH_2); 8.19 (bs, 2H, Ar–H); 7.56–7.52 (m, 2H, Ar–H); 7.35–7.14 (m, 11H, Ar–H); 5.47 (s, 2H, 1-CH_2Ph); 4.47 (d, 2H, J = 5.7 Hz, NHCH_2Ph). Anal. Calcd. for $C_{23}H_{20}N_2O$ (%): C, 81.15; H, 5.92; N, 8.23. Found: C, 81.29; H, 5.81; N, 8.44.

*6.1.3.2. 1-Benzyl-N-phenyl-1H-indole-3-carboxamide (**9b**).* ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 9.81 (s exch., 1H, NH); 8.42 (s, 1H, H-2); 8.24–8.20 (m, 1H, H-4); 7.79–7.75 (m, 2H, Ar–H); 7.60–7.55 (m, 1H, Ar–H); 7.33–7.00 (m, 10H, Ar–H); 5.53 (s, 2H, 1-CH_2Ph). Anal. Calcd. for $C_{22}H_{18}N_2O$ (%): C, 80.96; H, 5.56; N, 8.58. Found: C, 81.15; H, 5.47; N, 8.39.

*6.1.4. Synthesis of 1-benzyl-1H-indole (**15**)*

Indole **10** (1.7 g, 14.5 mmol) was added to a stirred solution of NaOH (1.2 g, 30 mmol) in DMSO (5 mL); the mixture was stirred for 15 min. After this time benzylchloride was added drop wise to the cooled (15 °C) mixture. The reaction mixture was stirred for 3 h, added with ice/water (200 mL) to give a solid that was collected by filtration and washed with water until neutral pH. Yield 80%, mp 41–42 °C (lit. [47]: mp 41–43 °C; lit. [48]: mp 43 °C).

6.2. Biology

6.2.1. Adenosine receptor binding assay. Materials

[^{3}H]DPCPX, [^{3}H]NECA, and [^{125}I]AB-MECA were obtained from DuPont-NEN (Boston, MA). ADA was from Sigma Chemical Co. (St. Louis, MO). All other reagents were from standard commercial sources and of the highest commercially available grade. CHO cells stably expressing human A_1, A_{2A}, and A_3 ARs were kindly supplied by Prof. K.N. Klotz, Wurzburg University, Germany [49].

6.2.2. Human A_1 adenosine receptors

Aliquots of cell membranes (30 μg proteins) were incubated at 25 °C for 180 min in 500 μL of T_1 buffer (50 mM Tris–HCl, 2 mM $MgCl_2$, 2 units/mL ADA, pH 7.4) containing [^{3}H]DPCPX (3 nM) and six different concentrations of the newly synthesized compounds. Non-specific binding was determined in the presence of 50 μM R-PIA [35]. The dissociation constant (K_d) of [^{3}H]DPCPX in *h*A_1 CHO cell membranes was 3 nM.

6.2.3. Human A_{2A} adenosine receptors

Aliquots of cell membranes (30 μg) were incubated at 25 °C for 90 min in 500 μL of T_2 buffer (50 mM Tris–HCl, 2 mM $MgCl_2$, 2 units/mL ADA, pH 7.4) in the presence of 30 nM of [^{3}H]NECA and six different concentrations of the newly synthesized compounds. Non-specific binding was determined in the presence of 100 μM R-PIA [35]. The dissociation constant (K_d) of [^{3}H]NECA in *h*A_{2A} CHO cell membranes was 30 nM.

6.2.4. Human A_3 adenosine receptors

Aliquots of cell membranes (30 μg) were incubated at 25 °C for 90 min in 100 μL of T_3 buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 units/mL ADA, pH 7.4) in the presence of 1.4 nM [^{125}I]AB-MECA and six different concentrations of the newly synthesized compounds. Non-specific binding was determined in the presence of 50 μM R-PIA [35]. The dissociation constant (K_d) of [^{125}I]AB-MECA in *h*A_3 CHO cell membranes was 1.4 nM.

All compounds were routinely dissolved in DMSO and diluted with assay buffer to the final concentration, where the amount of DMSO never exceeded 2%. Percentage inhibition values of specific radiolabelled ligand binding at 1–10 μM concentration are means ± SEM of at least three determinations.

6.2.5. Measurement of cyclic AMP levels on human A_1, and A_{2B} AR-transfected CHO cells

Intracellular cyclic AMP (cAMP) levels were measured using a competitive protein binding method [50]. 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 adenosine deaminase (ADA) (1 U/mL) and the phosphodiesterase inhibitor Ro20-1724 (20 μM). The pharmacological profile of the compounds towards 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 ARs was evaluated by assessing their ability to counteract NECA-mediated inhibition of cAMP accumulation in the presence of 1 μM forskolin, as non-selective adenylate cyclase (AC) activator. Cells were incubated in the reaction medium (15 min at 37 °C) with different concentrations of the target compound (1 nM–10 μM) 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 rpm 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. 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. The radioactivity was measured by liquid scintillation spectrometry.

6.2.6. Data analysis

All binding and functional data were analyzed using the non-linear regression curve fitting program GraphPad, version 5.0. EC_{50}, IC_{50} and K_i values were directly obtained from the dose response curves. All values obtained are mean values of at least three different experiments each performed in duplicate.

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