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Pharmacological characterization of novel adenosine ligands in recombinant and native human A_{2B} receptors

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Abstract

The present study was designed to evaluate the effects of novel and recognised compounds at human recombinant A_{2B} adenosine receptors expressed in Chinese hamster ovary (hA_{2B}CHO), in human embryonic kidney 293 (hA_{2B}HEK-293) and at endogenous A_{2B} receptors in human mast cells (HMC-1). Saturation binding experiments performed using the new high affinity A_{2B} adenosine radioligand [^{3}H]-*N*-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetra hydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide ([^{3}H]-MRE 2029F20) revealed a single class of binding sites in hA_{2B}CHO, hA_{2B}HEK-293 and HMC-1 cells with K_D (nM) of 1.65 ± 0.18, 2.83 ± 0.34, 2.62 ± 0.27 and B_{max} (fmol/mg protein) of 36 ± 4, 475 ± 50 and 128 ± 15, respectively. The pharmacological profile of new compounds, determined in inhibition binding experiments in hA_{2B}HEK-293 cells using [^{3}H]-MRE 2029F20, showed a rank order of potency typical of the A_{2B} receptors with K_i values in the range 3.2–28 nM. In functional assays, recognised agonists and antagonists were studied by evaluating their capability to modulate the cAMP production in hA_{2B}CHO and in HMC-1 cells. Novel compounds were able to decrease NECA-stimulated cAMP production in hA_{2B}CHO and in HMC-1 cells showing a high potency. New compounds were also able to inhibit cAMP levels in the absence of NECA and in the presence of forskolin stimulation in hA_{2B}CHO and in

Abbreviations: AS16, 2-(4-benzyloxy-phenyl)-*N*-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yl]-acetamide; AS70, 8-[2-methyl-5-[2-oxo-2(4-phenyl-piperazin-1-yl)-ethoxy]-2H-pyrazol-3-yl]-1,3-dipropyl-3,7-dihydro-purine-2,6-dione; AS74, *N*-benzo-[1,3]-dioxol-5-yl-2[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-isoxazol-3-yloxy]-acetamide; AS94, *N*-[4-(ethoxycarbonyl)phenyl]2-[5-(2,6-dioxo-1,3dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide; AS95, N-(4-carboxy-phenyl)-2-[5-(2,6-dioxo-1,3dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide; AS96, 1,3-diallyl-8-[2-methyl-5-[2-oxo-2(4-phenyl-piperazin-1-yl)-ethoxy]-2H-pyrazol-3-yl]-3,7-dihydro-purine-2,6-dione; AS99, *N*-[3,4] (dimethyl-phenyl)2-[5-(2,6-dioxo-1,3dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide; AS100, *N*-2,3,4 dichlorophenyl)-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]acetamide; AS101, *N*-(3,4-dimethoxy-phenyl)-2-5-(2,6-di-oxo-1,3-dipropyl-2,3,6,7-tetra-hydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide; cAMP, cyclic AMP; CGS 21680, 2-[*p*-(2-carboxyethyl)-phenetyl-amino]-5′-*N*-ethyl-carboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; HE-NECA, 2-hexynyl-5′-*N*-ethylcarboxamidoadenosine; [^{3}H]-MRE 2029F20, [^{3}H]-*N*-benzo [1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide; [^{3}H]-MRE 3008F20, [^{3}H]-5-*n*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; [^{3}H]-ZM 241385, [^{3}H]-4-(2-[7-amino-2-[furyl] [1,2,4] triazolo [2,3-a] [1,3,5]triazin-5-ylamino]ethyl] phenol; [^{3}H]-DPCPX; [^{3}H]-1,3-dipropyl-8-cyclopentyl-xanthine; hA_{2B}CHO, human A_{2B} adenosine receptor in Chinese hamster ovary; hA_{2B}-HEK-293, human A_{2B} adenosine receptor in human embryonic kidney 293; HMC-1, human mast cells; MRE 3008F20, 5-*n*-(4-methoxy phenyl-carbamoyl) amino-8-propyl-2-(2-furyl) pyrazolo[4,3-e]-1,2,4-triazolo [1,5-c] pyrimidine; MRE 2029F20, *N*-benzo[1,3]-dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide; NECA, 5′-*N*-ethylcarboxamidoadenosine; R-PIA, R(−)-N^6(2-phenyl-isopropyl)-denosine; S-PIA, S(−)-N^6(2-phenylisopropyl)-adenosine; SCH 58261, 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine

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HMC-1 cells. In HEK-293 cells MRE 2029F20 reduced cAMP basal levels with an IC_{50} value of 2.9 ± 0.3 nM. These results suggest that novel compounds are antagonists with an inverse agonist activity in recombinant and native human A_{2B} receptors.

Keywords: A_{2B} adenosine receptors; Inverse agonists; Cyclic AMP assays; hA_{2B}CHO cells; HEK-293 cells; HMC-1 cells

1. Introduction

Adenosine interacts with four cell surface receptor subtypes, A_1, A_{2A}, A_{2B} and A_3, which are coupled to different G-proteins [1]. From a functional point of view, A_{2A} and A_{2B} receptors typically respond with an increase in cyclic AMP suggesting that they are an example of Gs-coupled receptors [2,3]. A_{2B} receptor activation also elevates IP_3 levels, indicating this receptor can couple also to Gq-proteins [4]. A_{2B} receptors have been implicated in the regulation of mast cell secretion [5,6], gene expression [7,8], intestinal function [9], neurosecretion [10], vascular tone [11] and in particular asthma [3]. In the last few years, significant advancement has been made in the knowledge of the molecular pharmacology of the A_1, A_{2A} and A_3 adenosine receptors through the use of highly potent and selective agonists and/or antagonists, but the study of A_{2B} receptor has been hampered until now due to the lack of selective ligands. Recently, high affinity radioligands for A_{2B} receptors have been described such as [^{3}H]-MRS 1754 and [^{3}H]OSIP339391 showing affinity values in the nanomolar range [12,13]. A new selective antagonist radioligand [^{3}H]-MRE 2029F20, that is able to bind human A_{2B} adenosine receptors with an affinity value in the low nanomolar range, has been also synthesised and pharmacologically characterized [14,15]. Until now the characterization of A_{2B} receptor function has often been complicated by the fact that all adenosine receptor subtypes are frequently co-expressed in the same cells. Simultaneous expression of A_{2A}, A_{2B} and A_3 receptors has been found in pheochromocytoma PC12 cells, Jurkat leukaemia cells and human mast cell line HMC-1 [5,16,17]. Therefore, the study of potential new drugs on cell lines expressing multiple subtypes would be aided by the use of potent and selective A_{2B} ligands. It is well known that A_{2B} receptor stimulation enhances the release of pro-inflammatory mediators, and as a consequence, the search of new and potent A_{2B} antagonists may represent a novel target for the treatment of the diseases characterized by marked inflammatory state [3,18]. From a pharmacological point of view, it is well known that antagonists can be classified as neutral antagonists or antagonists with inverse agonist activity based on their effectiveness to reduce the agonist-independent activity of receptors [19,20]. In particular, antagonists that reduce the level of agonist-independent functional responses are called inverse agonists or antagonists with negative intrinsic activity whereas antagonists that do not reduce activity are referred to as neutral antagonists [21,22]. Recently, it has been suggested that up to 85% of antagonists of G-protein-coupled receptors are actually inverse agonists and that only 15% would be true neutral antagonists [23].

The present paper describes the pharmacological characterization of novel A_{2B} ligands to assess their possible antagonists or inverse agonist properties by studying the modulation of the cyclic AMP production in hA_{2B}CHO, in HEK-293 and in HMC-1 cells. The results obtained indicate that new compounds are antagonists with an inverse agonist activity in recombinant and native human A_{2B} adenosine receptors. Furthermore, the development of potent and selective A_{2B} adenosine compounds appears particularly promising to increase the knowledge of the potential role of A_{2B} receptors in the pathogenesis of several disorders. These new A_{2B} inverse agonists may become useful tools in human diseases characterized by a marked inflammatory component and could represent lead compounds for future drug development.

2. Materials and methods

2.1. Materials

NECA, HE-NECA, R-PIA, S-PIA, CGS 21680, theophylline, DPCPX, cAMP, Ro 20-1724, adenosine deaminase were obtained from Sigma-RBI (St. Louis, MO, USA). ZM 241385 and [^{3}H]-ZM 241385 (specific activity 17 Ci/mmol) were obtained from Tocris Cookson Ltd. (Bristol, UK). SCH 58261, MRE 2029F20, AS16, AS70, AS74, AS94, AS95, AS96, AS99, AS100, AS101 and MRE 3008F20 were synthesized by Prof. Pier Giovanni Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Italy). [^{3}H]-MRE 2029F20 (specific activity 123 Ci/mmol) and [^{3}H]-MRE 3008F20 (specific activity 67 Ci/mmol) derived from Amersham International Chemical Laboratories (Buckinghamshire, UK). [^{3}H]-DPCPX (specific activity 120 Ci/mmol), [^{3}H]-cAMP (specific activity 21 Ci/mmol) were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). HMC-1 cells were provided from Dr. J.H. Butterfield (Mayo Clinic). All other reagents were of analytical grade and obtained from commercial sources.

2.2. Synthesis of novel compounds

The examined compounds were prepared as previously described [24] and the synthetic routes for their preparation were reported in Fig. 1. Briefly, the preparation of the target

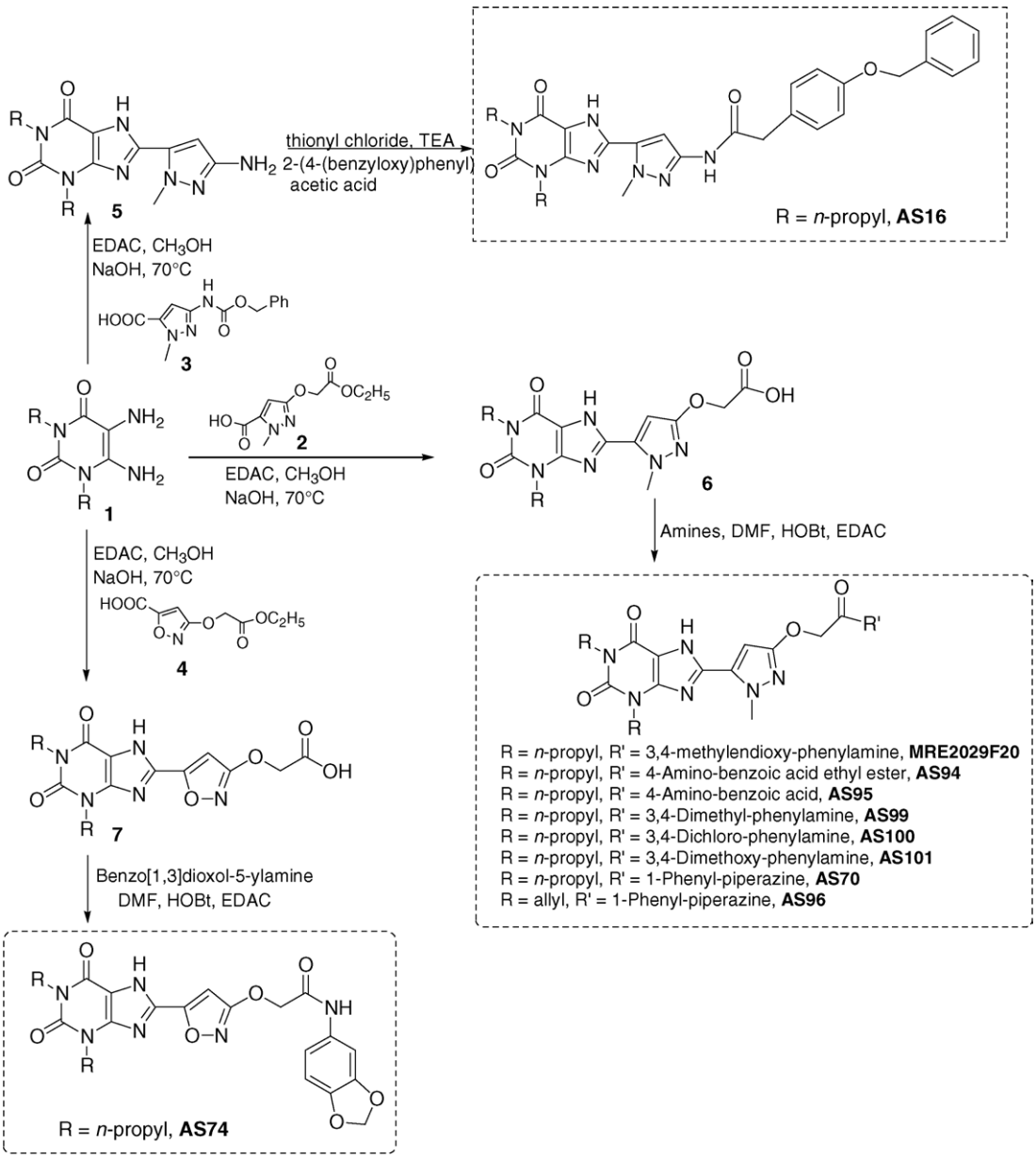

Fig. 1. Synthetic routes for the preparation of novel adenosine compounds.

compounds was achieved by a two-step sequence involving reaction between 1,3-disubsituted-5,6-diaminouracils **1** and the corresponding 5-ethoxycarbonylmethoxy-2-methyl-2*H*-pyrazole-3-carboxylic acid **2**, 5-benzyloxycarbonylamino-2-methyl-2*H*-pyrazole-3-carboxylic acid **3** or 3-ethoxycarbonylmethoxy-isoxazole-5-carboxylic acid **4** in methanol solution using 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDAC) as condensing agent, followed by ring closure in the presence of sodium hydroxide at 70 °C (Fig. 1). Xanthine amide derivative (AS16) was prepared by a coupling reaction among the amine derivatives **5** and the appropriate phenylacetic acid chloride obtained in turn, in presence of thionyl chloride and triethylamine in dichloromethane. Oxyacetamide xanthine derivatives (MRE 2029F20, AS70, AS74, AS94, AS95, AS96, AS99, AS100, AS101) were synthesised through the condensation of the appropriate carboxylic acid intermediates **6**, **7** with substituted anilines or *N*-phenyl piperazine in presence of EDAC and 1-hydroxy benzotrizole (HOBt) in DMF at room temperature.

2.3. Cell culture and membrane preparation

The transfection of CHO cells with hA_1, hA_{2A}, hA_{2B} and hA_3 adenosine receptors has been previously described [25]. Briefly, CHO and HEK-293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM/F12) with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), geneticin (G418, 0.2 mg/ml; A_{2B}, 0.5 mg/ml) at 37 °C in 5% CO_2/95% air until the use in cAMP assays. HMC-1 cells were maintained in Iscove's medium supplemented

with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1.2 mM α-thioglycerol until the use in cAMP assays. Similar experimental conditions were used to obtain CHO and HEK-293 membranes [26,15]. In particular for membrane preparation the culture medium was removed and the cell suspension was homogenized and centrifuged for 10 min at 1000 × *g*. The supernatant was then centrifuged again for 30 min at 100,000 × *g* and the membrane pellet was frozen at −80 °C until the use in saturation and competition binding experiments.

2.4. Saturation binding experiments on hA_{2B}HEK, hA_{2B}CHO and HMC-1 membranes

Saturation experiments of [^{3}H]-MRE 2029F20 (0.02–20 nM) to hA_{2B}HEK, hA_{2B}CHO and HMC-1 membranes were performed by incubating 100 μg of protein/assay of membrane suspension for 60 min at 4 °C. Non-specific binding was determined in the presence of 1 μM of MRE 2029F20 [14,15]. Saturation experiments on HMC-1 membranes were performed using [^{3}H]-DPCPX, [^{3}H]-ZM 241385 and [^{3}H]-MRE 300820 to study the presence of A_1, A_{2A} and A_3 adenosine receptor subtypes, respectively [26]. Briefly, these radioligands (0.01–10 nM) were incubated with 100 μg of protein/assay of membrane suspension for 90 min at 25 °C, 60 min at 4 °C and 150 min at 4 °C, respectively. Non-specific binding was determined in the presence of 1 μM of DPCPX, 1 μM of ZM 241385, 1 μM of MRE 3008F20, respectively. Bound and free radioactivities were separated by rapid filtration through Whatman GF/B glass–fiber filters using a Brandel instrument. The filter bound radioactivity was counted on a Scintillation Counter Tri Carb Packard 2500 TR (efficiency 57%). The protein concentration was determined according to a Bio-Rad method [27] with bovine albumin as reference standard.

2.5. Competition binding experiments at hA_1, hA_{2A}, hA_{2B} and hA_3 receptors

Binding of [^{3}H]-DPCPX to hA_1CHO membranes was performed for 120 min at 25 °C in 50 mM Tris–HCl buffer pH 7.4 containing 1 nM [^{3}H]-DPCPX, diluted membranes (100 μg of protein/assay) and at least six to eight different concentrations of antagonists were studied. Non-specific binding was determined in the presence of 1 μM of DPCPX and was always ≤10% of the total binding [24]. Binding of [^{3}H]-ZM 241385 to hA_{2A}CHO membranes (50 μg of protein/assay) was performed using 50 mM Tris–HCl buffer, 10 mM $MgCl_2$ pH 7.4 and at least six to eight different concentrations of antagonists were studied for an incubation time of 60 min at 4 °C. Non-specific binding was determined in the presence of 1 μM ZM 241385 and was about 20% of total binding [24]. Binding of [^{3}H]-MRE 3008F20 to hA_3CHO membranes were carried out in 50 mM Tris–HCl buffer, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4 containing 1 nM [^{3}H]-MRE 3008F20, diluted membranes (100 μg of protein/assay) and at least six to eight different concentrations of examined antagonists for 120 min at 4 °C. Non-specific binding was defined as binding in the presence of 1 μM MRE 3008F20 and was about 25–30% of total binding [26]. Competition experiments were performed in hA_{2B}-HEK membranes using 3 nM [^{3}H]-MRE 2029F20 and six to eight different concentrations of adenosine receptor ligands for an incubation time of 60 min at 4 °C. Non-specific binding was defined as binding in the presence of 1 μM MRE 2029F20 [15].

2.6. Measurement of cyclic AMP levels

CHO cells expressing hA_{2B} were suspended in 0.5 ml incubation mixture containing NaCl 150 mM, KCl 2.7 mM, NaH_2PO_4 0.37 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, glucose 5 mM, Hepes 5 mM, $MgCl_2$ 10 mM, pH 7.4 at 37 °C. Then 2.0 IU adenosine deaminase/ml and 0.5 mM Ro 20-1724 as phosphodiesterase inhibitor were added and pre-incubated for 10 min in a shaking bath at 37 °C. Adenosine receptor agonists such as HE-NECA, NECA, R-PIA, S-PIA, CGS 21680 (1 nM to 10 μM) were incubated for 10 min with the aim to evaluate their ability to stimulate adenylyl cyclase activity. Adenosine antagonists (theophylline, SCH 58261, MRE 3008F20) and novel pyrazolo derivatives (MRE 2029F20, AS16, AS70, AS74, AS94, AS95, AS96, AS99, AS100, AS101) at different concentrations (1 nM to 1 μM) were incubated for 10 min before the addiction of 100 nM NECA to evaluate the inhibition of cAMP accumulation. Moreover, the effect of the same ligands to modulate cAMP production in the presence of 2.0 IU adenosine deaminase/ml and 1 μM forskolin was tested. Similar experimental conditions were performed in HMC-1 cells with the aim to evaluate, in native cells, the effect of the same ligands on adenylate cyclase activity and on cAMP production. In hA_{2B}CHO cells, the effect of pertussis toxin at the final concentration of 200 ng/ml for 18 h on the modulation of cAMP levels were evaluated in the presence of 1 μM forskolin and 1 μM MRE 2029F20. We have also compared in hA_{2B}-CHO and HEK-293 cells the effect of 1 μM MRE 2029F20 on cAMP levels in the absence of ADA. We have also evaluated in HEK-293 cells the effect of MRE 2029F20 in the range 1 nM to 1 μM on cAMP basal levels to better investigate the inverse agonism behaviour. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay [26]. At the end of the incubation time (150 min at 4 °C) and after the addition of charcoal the samples were centrifuged at 2000 × *g* for 10 min. The clear supernatant was mixed with 4 ml of scintillator (Atomlight) and counted in a Tri Carb Packard 2500 TR scintillation counter.

Table 1
Affinity and potency of recognised adenosine agonists in hA_{2B}HEK-293 membranes and in hA_{2B}CHO and HMC cells

Agonists	[^{3}H]-MRE 2029F20 binding, K_i (nM) hA_{2B}HEK-293	cAMP assay EC_{50} (nM) hA_{2B}CHO	cAMP assay EC_{50} (nM) HMC-1
NECA	260 ± 30	152 ± 6	420 ± 50
HE-NECA	450 ± 52	254 ± 15	600 ± 80
R-PIA	3200 ± 380	2300 ± 250	3500 ± 400
S-PIA	5800 ± 645	4400 ± 500	6100 ± 700
CGS 21680	>10000	>10000	60 ± 8

Data are mean ± S.E.M. from four independent experiments. Inhibition binding experiments and cAMP assays were performed as described in Section 2. K_i value represents the concentration of drug able to displace 50% of the radioligand; EC_{50} is the concentration of agonist that stimulates the 50% of the maximal effect.

2.7. Data analysis

Saturation and competition binding experiments were analysed with the program LIGAND [28], which performs weighted, non-linear, least squares curve fitting program. EC_{50} and IC_{50} values obtained in cyclic AMP assay were calculated by non-linear regression analysis using the equation for a sigmoid concentration–response curve (GraphPad Prism, San Diego, CA, USA). K_B value was calculated using a derivation of Cheng–Prusoff equation [29]. All experimental data are expressed as mean ± standard error of the mean (S.E.M.) of three or four independent experiments performed in duplicate.

3. Results

3.1. Saturation binding assays to human adenosine receptors

[^{3}H]-MRE 2029F20 saturation experiments in hA_{2B}-CHO, hA_{2B}HEK-293 and in HMC-1 membranes showed an affinity expressed as K_D of 1.65 ± 0.18, 2.83 ± 0.34, 2.62 ± 0.27 nM, respectively. On the contrary, the receptor density was quite different in hA_{2B}CHO, hA_{2B}HEK-293 and in HMC-1 membranes with B_{max} values of 36 ± 4, 475 ± 50, 128 ± 15 fmol/mg protein, respectively. The expression of A_1, A_{2A} and A_3 adenosine receptors in HMC-1 membranes was determined using [^{3}H]-DPCPX, [^{3}H]-ZM 241385 and [^{3}H]-MRE 3008F20, respectively. Saturation of [^{3}H]-ZM 241385 binding to A_{2A} receptors displayed a K_D value of 1.92 ± 0.22 nM and a B_{max} value of 72 ± 8 fmol/mg protein. [^{3}H]-MRE 3008F20 binding experiments exhibited high affinity for A_3 receptors with a K_D of 1.53 ± 0.19 nM and a B_{max} value of 310 ± 35 fmol/mg protein. On the contrary [^{3}H]-DPCPX binding to A_1 receptors was undetectable in HMC-1 membranes. Computer analysis of the data failed to show a significantly better fit to a two-site as compared with a one-site binding model indicating that only one class of high affinity binding sites was present under our experimental conditions. The comparison of fits of the saturation binding curves to models with both one and two binding sites showed a not significant F-ratio in the range from 1.05 to 1.27.

3.2. Competition binding assays to human adenosine receptors

Table 1 reports the A_{2B} affinity values of the examined agonists obtained in [^{3}H]-MRE 2029F20 competition binding experiments performed in hA_{2B}HEK-293 membranes. The order of potency of the agonists was as follows: NECA > HE-NECA > R-PIA > S-PIA > CGS 21680. Tables 2 and 3 show the affinity versus A_1, A_{2A}, A_{2B} and A_3 adenosine receptor subtypes, expressed as K_i, and selectivity of recognised and novel compounds. The order of potency versus A_{2B} receptors of the antagonists examined was: MRE 2029F20 ≥ AS101 ≥ AS99 > AS100 > AS70 > AS96 > AS94 ≥ AS95 > AS16 > AS74 > MRE 3008F20 > theophylline > SCH 58261 (Tables 2 and 3). New compounds displayed an high affinity to hA_{2B} receptors showing K_i values in the range 3.2–28 nM. AS16, AS74, AS94, AS95, AS96 and AS101 did not interact with hA_1, hA_{2A} and hA_3 receptors (K_i > 1 μM). MRE 2029F20, AS70, AS99 and AS100 showed a weak affinity versus hA_1 receptors with K_i values in the high nanomolar range (K_i = 200–750 nM) (Table 3). Moreover, novel compounds revealed a good selectivity for human A_{2B} receptors. In particular, MRE 2029F20 the most potent compound of this series at hA_{2B} receptors, showed a good selectivity versus other receptor subtypes (A_1/A_{2B} = 63, A_{2A}/A_{2B} = A_3/A_{2B} > 312). We have also studied recognised adenosine antagonists such as MRE 3008F20 that exhibited high affinity and selectivity versus hA_3 receptors and low affinity for hA_{2B} receptors. The binding affinity of SCH 58261 to hA_1CHO membranes was lower (K_i = 520 nM) than that observed in hA_{2A}CHO membranes (K_i = 1.5 nM) resulting in a high A_{2A} selectivity. SCH 58261 was the only compound that did not interact with hA_{2B} and hA_3 receptors. Theophylline was found to be a poor adenosine antagonist with a very low affinity in the micromolar range versus all adenosine receptor subtypes suggesting that this antagonist is not A_{2B} selective (Table 3).

Table 2
Affinity and potency of recognised and novel adenosine antagonists in hA_{2B}HEK-293 membranes, in hA_{2B}CHO and in HMC-1 cells

Antagonists	[^{3}H]-MRE 2029F20 binding, K_i (nM) hA_{2B}HEK-293	cAMP assay + NECA K_B (nM) hA_{2B}CHO	cAMP assay + NECA K_B (nM) HMC-1	cAMP assay + forskolin IC_{50} (nM) hA_{2B}CHO	cAMP assay + forskolin IC_{50} (nM) HMC-1
MRE 2029F20	3.2 ± 0.3	23 ± 3	19 ± 2	0.05 ± 0.01	0.08 ± 0.01
AS101	3.8 ± 0.4	27 ± 3	28 ± 3	0.42 ± 0.05	0.86 ± 0.12
AS99	4.2 ± 0.5	29 ± 4	32 ± 4	0.53 ± 0.06	1.01 ± 0.15
AS100	6.0 ± 0.7	37 ± 5	38 ± 5	0.64 ± 0.06	1.32 ± 0.14
AS70	8.1 ± 0.9	44 ± 4	47 ± 5	0.61 ± 0.07	1.28 ± 0.13
AS96	11 ± 2	47 ± 5	49 ± 5	0.72 ± 0.07	1.43 ± 0.15
AS94	17 ± 2	68 ± 6	72 ± 8	0.75 ± 0.08	1.64 ± 0.17
AS95	19 ± 2	71 ± 7	74 ± 7	0.84 ± 0.09	1.72 ± 0.18
AS16	22 ± 3	75 ± 9	87 ± 9	0.95 ± 0.12	1.89 ± 0.21
AS74	28 ± 3	84 ± 9	93 ± 10	1.27 ± 0.10	2.21 ± 0.24
MRE 3008F20	1500 ± 165	>10000	>10000	>10000	>10000
Theophylline	4800 ± 520	>10000	>10000	>10000	>10000
SCH 58261	>10000	>10000	>10000	>10000	>10000

Inhibition binding experiments and cAMP assays were described in Section 2. cAMP experiments were performed using as stimulation of cAMP levels NECA (100 nM) in hA_{2B}CHO, NECA (400 nM) in HMC-1 cells and forskolin 1 μM in both cell lines. Data are mean ± S.E.M. ($n = 4$). K_i value represents the concentration of drug able to displace 50% of the radioligand; IC_{50} is the concentration of antagonist that reduces the response by 50% of the maximal effect; K_B value was calculated by IC_{50} corrected using the derivation of Cheng–Prusoff equation.

3.3. Effect of adenosine agonists on cAMP levels in hA_{2B}CHO and HMC-1 cells

All adenosine analogues were able to stimulate adenylyl cyclase activity and to increase cAMP levels in hA_{2B}CHO cells (Fig. 2A). NECA appeared to be the most potent agonist (EC_{50} = 152 ± 6 nM) followed by HE-NECA (EC_{50} = 254 ± 15 nM), R-PIA (EC_{50} = 2300 ± 250 nM) and S-PIA (EC_{50} = 4400 ± 500 nM). CGS 21680 was not able to mediate an increase of cAMP levels in the range 1 nM to 10 μM (Table 1). In HMC-1 cells, NECA, HE-NECA, R-PIA and S-PIA showed a lower potency relative to hA_{2B}CHO cells with EC_{50} values in the range 420–6100 nM (Fig. 2B). The concentration–response relationship of the agonists for cAMP accumulation in HMC-1 cells followed a shallow curve with a Hill slope in the range 0.63–0.72 indicative of two receptors mediating agonist responses. Moreover, the A_{2A} agonist CGS 21680-stimulated cAMP production with an EC_{50} of 60 ± 8 nM (Hill slope 0.99 ± 0.08) confirming the presence of A_{2A} adenosine receptors in HMC-1 cells (Table 1; Fig. 2B). The selective A_{2A} antagonist SCH 58261 (1 μM) fully antagonized the rise in cAMP levels mediated by CGS 21680 (100 nM) that decreased cAMP production of 94 ± 5%. In addition, the stimulatory effect in HMC-1 cells of NECA, a non-selective adenosine agonist, was evaluated in the presence of selective antagonists to discriminate the role of the different adenosine receptor subtypes. The EC_{50} value of NECA calculated in the presence of 1 μM MRE 2029F20, and therefore, due to the A_{2A} stimulation, was 105 ± 12 nM (Hill slope = 1.01 ± 0.09). In addition, the EC_{50} value of NECA calculated in the presence of 1 μM SCH 58261 (A_{2A} antagonist) and due to the A_{2B} component was 1950 ± 207 nM (Hill slope = 0.98 ± 0.15) (Fig. 3A). We obtained the dose–response curve of NECA also in the presence of 10 μM CGS 21680 that completely

Table 3
Affinity versus A_1, A_{2A}, A_{2B} and A_3 adenosine receptor subtypes and selectivity of novel and known compounds

Antagonists	[^{3}H]-DPCPX binding, K_i (nM) hA_1CHO	[^{3}H]-ZM 241385 binding, K_i (nM) hA_{2A}CHO	[^{3}H]-MRE 2029F20 binding, K_i (nM) hA_{2B} HEK-293	[^{3}H]-MRE 3008F20 binding, K_i (nM) hA_3CHO	A_1/A_{2B}	A_{2A}/A_{2B}	A_3/A_{2B}
MRE 2029F20	200 ± 25	>1000	3.2 ± 0.3	>1000	63	>312	>312
AS101	>1000	>1000	3.8 ± 0.4	>1000	>263	>263	>263
AS99	750 ± 80	>1000	4.2 ± 0.5	>1000	179	>238	>238
AS100	300 ± 36	>1000	6.0 ± 0.7	>1000	50	>167	>167
AS70	250 ± 30	>1000	8.1 ± 0.9	>1000	31	>123	>123
AS96	>1000	>1000	11 ± 2	>1000	>91	>91	>91
AS94	>1000	>1000	17 ± 2	>1000	>59	>59	>59
AS95	>1000	>1000	19 ± 2	>1000	>53	>53	>53
AS16	>1000	>1000	22 ± 3	>1000	>45	>45	>45
AS74	>1000	>1000	28 ± 3	>1000	>36	>36	>36
MRE 3008F20	1120 ± 130	165 ± 18	1500 ± 165	0.9 ± 0.1	0.75	0.11	0.0006
Theophylline	6500 ± 720	2400 ± 350	4800 ± 520	>10000	1.4	0.5	>2
SCH 58261	520 ± 80	1.5 ± 0.3	>10000	>10000	<0.052	<0.00015	>1

Experimental conditions are described in Section 2. Results are means ± S.E.M. from four independent experiments.

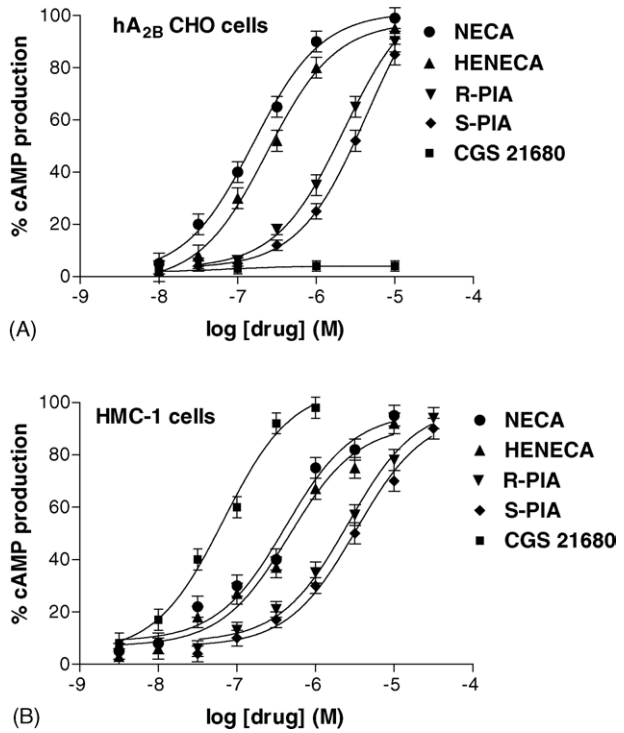

Fig. 2. Stimulation of cAMP levels in hA_{2B}CHO cells (A) and in HMC-1 cells (B) by adenosine agonists. Drug effects are expressed as a percentage of cAMP production. Data represent means ± S.E.M. of four experiments each performed in duplicate.

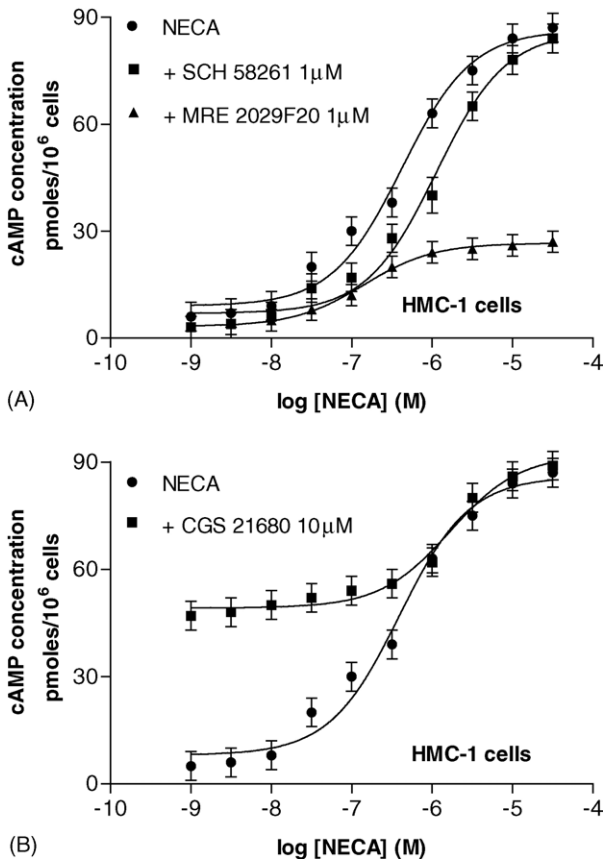

Fig. 3. (A) Stimulation of cAMP levels in HMC-1 cells by NECA in the absence and in the presence of MRE 2029F20 (1 μM) and of SCH 58261 (1 μM). (B) Stimulation of cAMP levels in HMC-1 cells by NECA in the absence and in the presence of CGS 21680 (10 μM). Data represent means ± S.E.M. of four experiments each performed in duplicate.

occupies A_{2A} receptors without having an effect at A_{2B} receptors. After saturation of A_{2A} receptors with CGS 21680 the dose–response curve of NECA showed an $EC_{50} = 1877 \pm 196$ nM with a Hill coefficient near to the unity (a result analogue to that obtained in the presence of SCH 58261) corresponding presumably to the activation of A_{2B} receptors (Fig. 3B). All experiments were performed in the presence of MRE 3008F20 (100 nM) to block A_3 adenosine receptors.

3.4. Effect of novel and known adenosine compounds on NECA-stimulated cAMP levels in hA_{2B}CHO and HMC-1 cells

In hA_{2B}CHO cells, all novel adenosine compounds were able to inhibit the stimulatory effect of 100 nM NECA with the following order of potency: MRE 2029F20 ≥ AS101 ≥ AS99 ≥ AS100 ≥ AS70 ≥ AS96 > AS94 ≥ AS95 ≥ AS16 ≥ AS74 (Table 2; Fig. 4). In particular, MRE 2029F20 was the best compound in hA_{2B}CHO with a K_B value of 23 ± 3 nM (Table 2). AS101, AS99 and AS100 showed a good potency in the nanomolar range in hA_{2B}CHO cells ($K_B = 27 \pm 3$; 29 ± 4; 37 ± 5 nM, respectively). In addition, also the other compounds examined had K_B values in the nanomolar range (from 44 to 84 nM). Interestingly, also in HMC-1 cells these compounds were able to block the stimulation mediated by NECA with a K_B in the range 19–93 nM (Table 2; Fig. 5).

MRE 2029F20, AS101 and AS99 had higher effect than the other novel adenosine compounds in both the examined cell lines (Table 2). In HMC-1 cells, novel compounds at 1 μM were able to only partially reduce the cAMP stimulation revealing that 20–27% of the cAMP accumulation is probably due to A_{2A} stimulation. The recognised A_{2A} and A_3 antagonists SCH 58261 and MRE 3008F20 were not able alone to inhibit the NECA-stimulation of cAMP levels in either cell lines (Table 2). Theophylline was not able to reduce cAMP production in hA_{2B}CHO and in HMC-1 cells in the range of investigated concentration 1 nM to 10 μM (Table 2). The Spearman's rank correlation between affinity values of [^{3}H]-MRE 2029F20 binding to hA_{2B}HEK-293 membranes and potency values on cAMP assay in hA_{2B}CHO and HMC-1 cells by novel compounds was 0.99 and 0.98, respectively ($P < 0.01$).

3.5. Effect of novel and known adenosine compounds on forskolin-stimulated cAMP levels in hA_{2B}CHO and HMC-1 cells

The effect of novel and recognised antagonists was evaluated in the presence of 1 μM forskolin to verify if

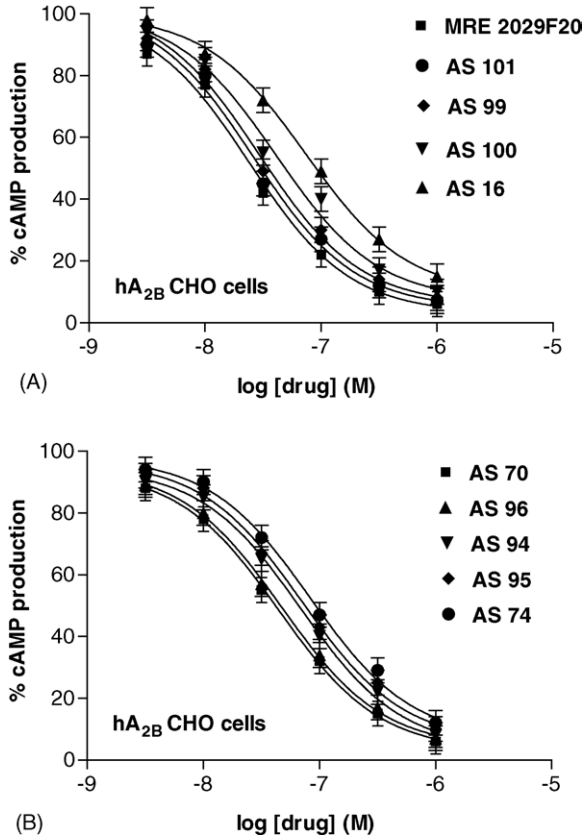

Fig. 4. (A and B) Inhibition of NECA-stimulated cAMP levels in hA_{2B}CHO cells by novel adenosine compounds. Data represent means ± S.E.M. of four independent experiments each performed in duplicate.

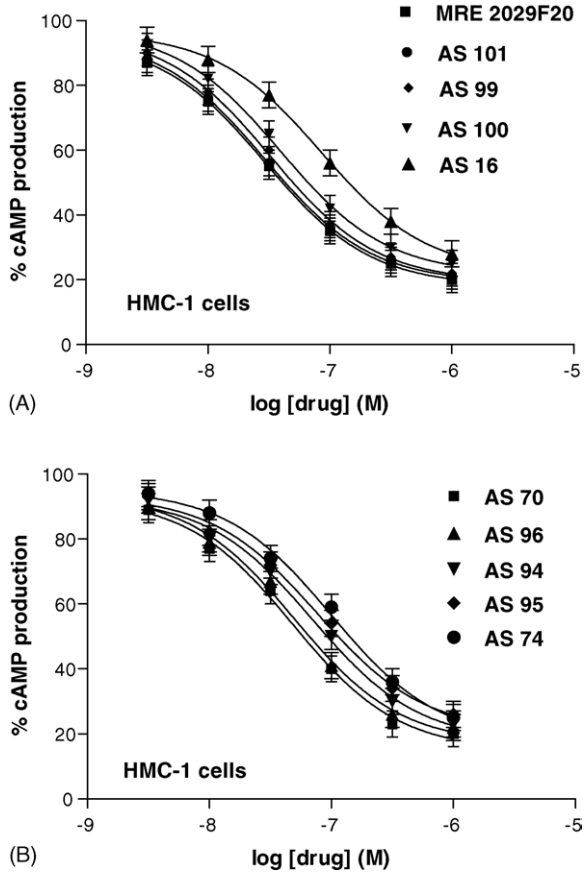

Fig. 5. (A and B) Inhibition of NECA-stimulated cAMP levels in HMC-1 cells by novel adenosine compounds. Data represent means ± S.E.M. of four independent experiments each performed in duplicate.

these compounds, in the absence of an agonist, mediated an inhibitory response (Figs. 6 and 7). This experimental condition was chosen to better identify a potential inverse agonist activity of the compounds. In the absence of forskolin, the basal level was very low (15–20 pmol cAMP/assay) hampering the evaluation of a direct inhibitory effect. In hA_{2B}CHO and in HMC-1 cells, theophylline, SCH 58261 and MRE 3008F20 were not able to modulate cAMP production (IC_{50} value > 10 μM). Interestingly, in each cell line and in the presence of forskolin 1 μM, all novel compounds at the concentration 1 μM were able to decrease cAMP levels by 30–50% (Table 2). The dose–response curves of the new compounds showed IC_{50} values in the 10^{-10} to 10^{-9} M range (50 pM–2 nM) with similar potency to block forskolin stimulation on cAMP levels in HMC-1 with respect to hA_{2B}CHO cells (Figs. 6 and 7). The order of potency of the novel compounds was strictly similar in both examined cell lines with the following order of potency: MRE 2029F20 > AS101 > AS99 > AS70 > AS100 > AS96 > AS94 > AS95 > AS16 > AS74. In hA_{2B}CHO cells, new compounds at the concentration of 1 μM were able to inhibit cAMP production of 40–50% (Fig. 6). Similarly, in HMC-1 cells novel compounds were also able to inhibit forskolin-stimulated cAMP production by 30–35% (Fig. 7) suggesting their role as A_{2B} inverse agonists in both cell preparation examined. The Spearman's rank correlation between affinity values of [^{3}H]-MRE 2029F20 binding to hA_{2B}HEK-293 membranes and potency values on cAMP assay in hA_{2B}CHO and HMC-1 cells by novel compounds was 0.89 and 0.88, respectively ($P < 0.01$). Finally, the Spearman's rank correlation of novel compounds calculated between potency values on cAMP assay modulated by NECA or by forskolin in hA_{2B}CHO and HMC-1 cells was 0.90 and 0.92, respectively ($P < 0.01$).

3.6. Effect of different experimental conditions on cAMP levels

The basal content (15 ± 2, 12 ± 3 and 16 ± 3 pmol × 10^6 cells in CHO wild type, hA_{2B}CHO and in HMC-1 cells, respectively) of cAMP was evaluated in the presence of adenosine deaminase (2 units/ml). Consequently, forskolin (1 μM), an activator of adenylyl cyclase and Ro 20-1724 (0.5 mM), an inhibitor of cAMP phosphodiesterase, were used to increase intracellular cAMP content.

In CHO wild type and hA_{2B}CHO cells, forskolin increased cAMP levels of 4.3 ± 0.4 and 6.3 ± 0.5 fold respect to the basal content, respectively ($n = 4$ experiments). The low difference of cAMP increase revealed in

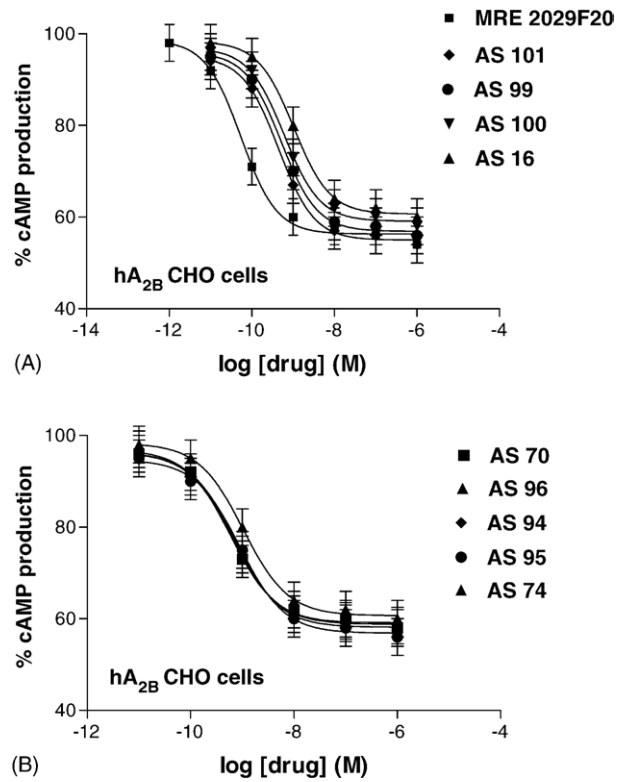

Fig. 6. (A and B) Inhibition of forskolin-stimulated cAMP levels in hA_{2B}CHO cells by novel adenosine compounds. Data represent means ± S.E.M. of four independent experiments each performed in duplicate.

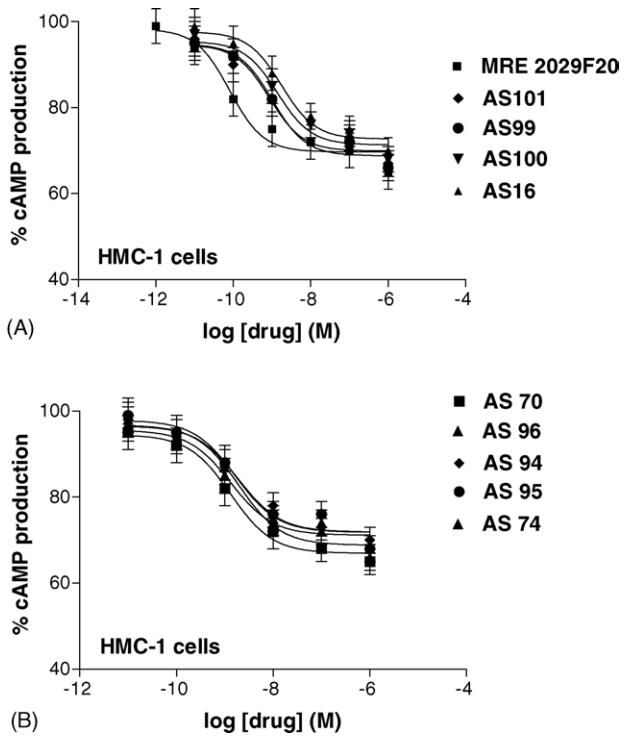

Fig. 7. (A and B) Inhibition of forskolin-stimulated cAMP levels in HMC-1 cells by novel adenosine compounds. Data represent means ± S.E.M. of four independent experiments each performed in duplicate.

these two cell lines is probably due to the low A_{2B} receptor density in CHO cells. Moreover, in CHO wild type cells, the effect of NECA and of the antagonists such as theophylline and MRE 2029F20 were studied ($n = 4$ experiments). In these cells NECA (100 nM), theophylline (10 μM) and MRE 2029F20 (1 μM) were not able to modulate cAMP levels determining 17 ± 2, 14 ± 3 and 13 ± 3 pmol $\times 10^6$ cells, respectively. The absence of the stimulation of cAMP levels in CHO wild type cells suggest that these compounds were not able to modulate cAMP levels in the absence of hA_{2B} receptors and did not interact directly with adenylate cyclase system.

In hA_{2B}CHO and in HEK-293 cells MRE 2029F20 (1 μM) was able to reduce the percentage of cAMP production, in the absence of ADA, of 42 ± 5 and $45 \pm 6\%$, respectively, suggesting that this compound competes with adenosine on A_{2B} receptor subtypes.

In addition, in hA_{2B}CHO cells NECA (100 nM) mediated an increase of adenylate cyclase activity with 60 ± 6 pmol $\times 10^6$ cells of cAMP that is reduced by the presence of theophylline (10 μM) and MRE 2029F20 (1 μM) to 37 ± 4 and 3.6 ± 0.4 pmol $\times 10^6$ cells, respectively ($n = 4$ experiments). Similarly, in HMC-1 cells NECA (400 nM) mediated an increase of adenylate cyclase activity with 55 ± 6 pmol $\times 10^6$ cells of cAMP that is reduced by the presence of theophylline (10 μM) and MRE 2029F20 (1 μM) to 44 ± 5 and 11 ± 1 pmol $\times 10^6$ ^{6} cells, respectively ($n = 4$ experiments).

In hA_{2B}CHO cells, no changes in cAMP production stimulated by forskolin (1 μM) and modulated by MRE 2029F20 (1 μM) were observed in the absence (65 ± 7 pmol $\times 10^6$ cells and 39 ± 3 pmol $\times 10^6$ cells, respectively) or in the presence of pertussis toxin (67 ± 6 pmol $\times 10^6$ cells and 41 ± 3 pmol $\times 10^6$ cells, respectively) that is able to inactivate Gi and Go family G-proteins ($n = 3$ experiments). These data demonstrated that the effect of MRE 2029F20 involves inverse agonism at Gs-protein without a contribution of Gi-protein.

Finally, the potency of MRE 2029F20 on cAMP basal levels in HEK-293 cells was evaluated using a 3-fold increased number of cells per assay with the aim to rise the pmol of cAMP in basal condition (50 ± 5 pmol $\times 3 \times 10^6$ cells). In this experimental condition, MRE 2029F20 was able to decrease in a statistically significant way ($P < 0.01$) cAMP levels showing an IC_{50} of 2.9 ± 0.3 nM (Fig. 8A). Fig. 8B compared the effect of MRE 2029F20 (1 μM) on cAMP accumulation in three different experimental conditions such as the basal levels, NECA (100 nM) and forskolin (1 μM) stimulation.

4. Discussion

The A_{2B} adenosine receptors represent a candidate for a large number of therapeutic applications due to their widespread distribution [2]. Activation of A_{2B} receptors would

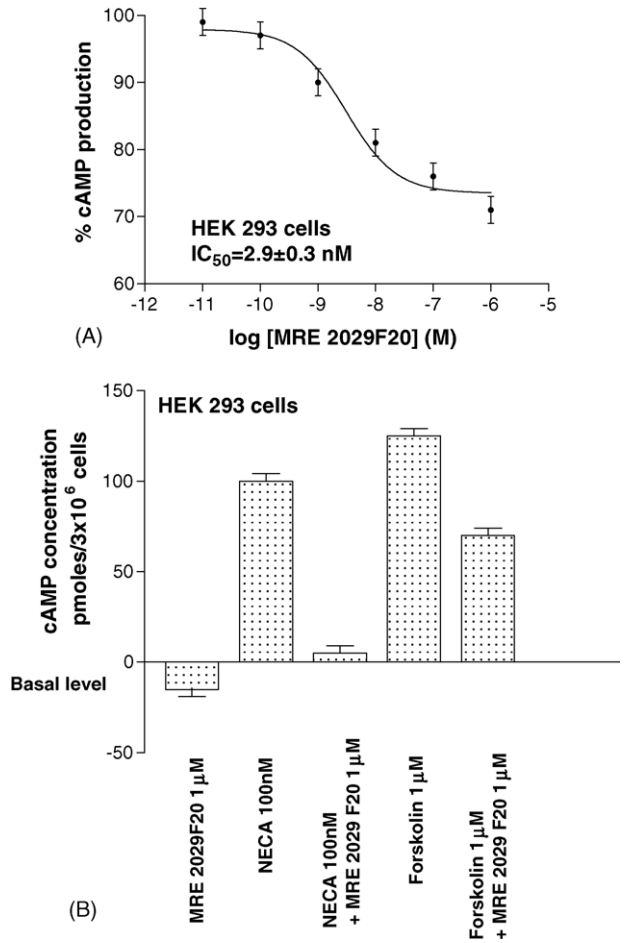

Fig. 8. (A) Inhibition of cAMP basal levels in HEK-293 cells by MRE 2029F20. (B) Effect of MRE 2029F20 (1 μM) in HEK-293 cells on cAMP basal levels, on NECA (100 nM) and forskolin (1 μM) stimulation.

contribute to the pathogenesis of inflammatory airways disease by acting on the HMC-1 cells that enhance the release of pro-inflammatory mediators [18]. The recognition of the potential role of all adenosine receptor subtypes in the chronic airway diseases raises the possibility that the modulation of these receptors may be a valuable therapeutic approach. In particular, the potential role of adenosine in the pathophysiology of asthma has suggested that the A_{2B} subtype may serve as a novel target for the treatment of this disease [30]. Theophylline, that is considered clinically useful for the treatment of asthma, shows low affinity and selectivity versus A_1, A_{2A}, A_{2B} and A_3 adenosine receptors. Moreover, blockade of A_{2A} receptors by theophylline may be counter-productive in the treatment of asthma because their activation has inhibitory effects on HMC-1 and several types of inflammatory cells [31]. As a consequence, the search for new selective and potent A_{2B} adenosine compounds may provide a more efficient and safer alternative to the use of theophylline in the treatment of these diseases.

It is now well established that G-protein-coupled receptors may exist in a spontaneously active state even in absence of agonists [22]. Antagonists that reduce the level of agonist-independent receptor activity and functional response are called inverse agonists or antagonists with negative intrinsic activity, whereas antagonists that do not reduce activity are referred to as neutral antagonists with no intrinsic activity [32]. It is now accepted that to study inverse agonism, G-protein-coupled receptors should possess a detectable level of constitutive activity. In addition, the modulation of a forskolin response has been shown to offer a useful method to investigate constitutive receptor activity in several cell lines [33]. Moreover, forskolin appears to activate adenylate cyclase through a unique mechanism involving both direct activation of the enzyme and facilitation or potentiation of the modulation of enzyme activity by receptors or the guanyl nucleotide binding subunit [34]. Several papers are present in the literature regarding many drugs with important therapeutic actions that had been assumed to be antagonists at G-protein-coupled receptors have now been shown to be inverse agonists in the same assay systems [35]. Several descriptions of inverse agonists rely on effects in recombinant systems and not to native tissues but recently some examples of inverse agonists in native systems have been described [36–39].

The present paper describes the effect of novel A_{2B} ligands by assessing their possible antagonist or inverse agonist properties using the modulation of cAMP production in hA_{2B}CHO and HMC-1 cells as examples of cells expressing recombinant (at a low density level) and native human A_{2B} adenosine receptors. Saturation binding experiments in hA_{2B}CHO and in HMC-1 cells revealed the presence of A_{2B} receptors, and in HMC-1 cells also found the expression of A_{2A} and A_3 receptors. Binding data clearly indicate the new compounds have higher affinity and selectivity for the human A_{2B} adenosine receptors with respect to recognised antagonists.

Well-known adenosine agonists have been studied evaluating their ability to increase the cAMP production in hA_{2B}CHO and in HMC-1 cells. Currently, no high-affinity agonists are available for the A_{2B} adenosine receptor with the only exception of NECA, which has good affinity and potency but not selectivity [24,12,13]. These literature data are in agreement in indicating NECA as the most potent A_{2B} agonist even if highly variable affinity or potency values are reported according to the specific experimental conditions used. The rank order of potency of the agonists to stimulate cAMP production is in perfect agreement with the affinities obtained in the binding assays and strictly similar in hA_{2B}CHO and in HMC-1 cells with the only exception of CGS 21680 that is inactive in the transfected cells but active in HMC-1 cells confirming the presence of A_{2A} receptors. With the aim of discerning the role of A_{2A} and A_{2B} receptors in HMC-1 cells we have studied the effect of NECA in the presence of high concentrations of CGS 21680 (A_{2A} agonist) or in the presence of SCH 58261 (A_{2A} antagonist). The results obtained are strictly similar and represent the potency of NECA in HMC-1 cells acting via A_{2B} adenosine receptors. We have also evaluated the

effect of NECA, in the presence of MRE 2029F20 to assess its potency to A_{2A} adenosine receptors. Our results indicate that the selective blockade of A_{2A} receptors with the antagonist SCH 58261 or the use of A_{2A} agonist CGS 21680 used together with the non-selective agonist NECA allows us to discriminate A_{2B} activation in the cells expressing both subtypes. Finally, the inhibitory effect of novel antagonists on cAMP production has been evaluated on hA_{2B}CHO and HMC-1 cells (Table 2). These compounds are able to inhibit the stimulatory effect of NECA on cAMP accumulation revealing a good correlation between K_B or IC_{50} values obtained in both the cell lines studied (Table 2). Our binding and functional data reveal high affinity values (K_i values), which are strictly correlated with potency estimates (K_B or IC_{50} values). Likewise, the Spearman's rank correlation coefficient between potency values in the cAMP assay modulated by NECA or by forskolin and receptor affinity values show a highly significant positive correlation. To evaluate if these compounds had an effect also in the absence of the agonist we performed cAMP assays in the presence of forskolin because in our experimental model, the basal level (12–18 pmol cAMP) is very low and hampered the evaluation of a further potential inhibitory effect. In hA_{2B}-CHO and in HEK-293 cells, MRE 2029F20 1 μM determines a reduction of cAMP levels in the presence of endogenous adenosine suggesting their specific competition versus A_{2B} receptors. In addition, pertussis toxin experiments demonstrated that the effect of MRE 2029F20 involves inverse agonism at Gs-protein without a contribution of Gi-protein. All new compounds show similar potency if studied in the presence of NECA or when tested against forskolin. Moreover, novel compounds show in HMC-1 cells IC_{50} values in the range 0.08–2.21 nM strictly similar to those observed in hA_{2B}CHO cells (IC_{50} values in the range 0.05–1.27 nM) (Table 2). These results demonstrate that new compounds are able to modulate cAMP production suggesting that they are antagonists with an inverse agonist activity in hA_{2B}CHO and in HMC-1 cells. Interestingly, these compounds show higher potency in cAMP assay with respect to the even high affinity found in binding experiments. We do not have a clear explanation for this phenomenon but we can remark that these assays have been performed using different cell lines and various experimental conditions. Alternatively, this difference could be due to various conformations of the receptor in these cell lines or to different receptor/G-protein-coupling. To better investigate the inverse agonism of MRE 2029F20 in HEK-293 cells it has been evaluated the inhibitory effect of this compound on cAMP basal levels. In the experimental condition of 3-fold increased number of cells MRE 2029F20 at the final concentration of 1 μM was able to decrease in a statistically significant way cAMP levels. The potency of MRE 2029F20 with an IC_{50} of 2.9 ± 0.3 nM suggests that this compound shows an A_{2B} inverse agonist behaviour.

In conclusion, when hA_{2B} receptors are expressed in CHO or in HEK-293 cells, constitutive activity of these receptors is manifested in the absence of agonist as suggested by the effect of novel ligands. Interestingly, these new compounds caused a decrease of forskolin-stimulated cAMP content in HMC-1 cells as an example of a cell line expressing native human A_{2B} receptors. MRE 2029F20 determined also a reduction of cAMP basal levels on HEK-293 cells expressing recombinant human A_{2B} receptors indicating the inverse agonist behaviour of these compounds in the cell lines examined.

As a consequence, the development of potent and selective A_{2B} adenosine compounds appears particularly promising to increase the knowledge of the potential role of A_{2B} receptors in the pathogenesis of several disorders. These new A_{2B} inverse agonists may become useful tools in human diseases characterized by a marked inflammatory component and could represent lead compounds for future drug development.

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