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A new chemical tool (C0036E08) supports the role of adenosine A_{2B} receptors in mediating human mast cell activation

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ABSTRACT

Asthma is a chronic inflammatory disease of the airways that involves many cell types, amongst which mast cells are known to be important. Adenosine, a potent bronchoconstricting agent, exerts its ability to modulate adenosine receptors of mast cells thereby potentiating derived mediator release, histamine being one of the first mediators to be released. The heterogeneity of sources of mast cells and the lack of highly potent ligands selective for the different adenosine receptor subtypes have been important hurdles in this area of research. In the present study we describe compound C0036E08, a novel ligand that has high affinity (pK_i 8.46) for adenosine A_{2B} receptors, being 9 times, 1412 times and 3090 times more selective for A_{2B} receptors than for A_1, A_{2A} and A_3 receptors, respectively. Compound C0036E08 showed antagonist activity at recombinant and native adenosine receptors, and it was able to fully block NECA-induced histamine release in freshly isolated mast cells from human bronchoalveolar fluid. C0036E08 has been shown to be a valuable tool for the identification of adenosine A_{2B} receptors as the adenosine receptors responsible for the NECA-induced response in human mast cells. Considering the increasing interest of A_{2B} receptors as a therapeutic target in asthma, this chemical tool might provide a base for the development of new anti-asthmatic drugs.

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Abbreviations: AMP, adenosine 5′monophosphate; BALF, bronchoalveolar fluid; CGS15943, 9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine; GM-CSF, granulocyte macrophage-colony stimulating factor; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; FceRI, high-affinity human IgE receptors; HMC, human mast cells; IgE, immunoglobulin E; IL, interleukin; IFN-γ, interferon γ; MRS, 1220 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino][1,2,4]-triazolo[1,5-c]quinazoline; MRS, 1754 8-[4-[((4-cyanophenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(*n*-propyl)xanthine; NECA, 5′-*N*-ethylcarboxamide-adenosine; PCA, perchloric acid; R-PIA, R(−) N6-(2-phenylisopropyl)-adenosine; SCH-58261, 5-amino-2-(2-furyl)-7-phenyilethyl-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; 8-SPT, 8-(*p*-sulfophenyl)-theophylline; TNF-α, tumor necrosis factor-alpha; XAC, 8-[-4-[[[[(2-aminoethyl)-amino]carbonyl]ethyl]oxy]phenyl]-1,3-dipropylxanthine; ZM241385, 4-(2-[7-amino-2-[furyl]-[1,2,4]triazolo[2,3-a]{1,3,5}triazin-5yl-amino]ethyl)phenol.

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1. Introduction

Asthma is a chronic inflammatory disease of the airways that involves many inflammatory and structural cell types (e.g. mast cells, eosinophils, basophils and lymphocytes). Activation of these cells results in the release of various inflammatory mediators that contribute to the typical pathophysiological processes that lead to the manifestation of asthma [1]. Mast cells, in particular, play a pivotal role in this system (for review see [1,2]). These cells possess high-affinity IgE receptors (FceRI) on their surface, and in the acute phase of the disease, the cross-linking of mast cell-bound IgE by allergens induces their activation and degranulation, whereby they release proinflammatory mediators. These mediators, such as histamine, adenosine, proteoglycans and leukotrienes contribute to the different components of the asthmatic response: bronchoconstriction, plasma exudation and mucus hypersecretion. In the chronic phase of asthma, mast cells also secrete a wide array of cytokines and growth factors including IL-1, IL-2, IL-3, IL-4, IL-5, IL-8, IL-9, IL-13, IFN-γ, TNF-α and GM-CSF [3]. These secretions contribute to the chronic inflammatory changes that take place, including fibrosis, hypertrophy and hyperplasia of the smooth muscle layer of the airway, hyperplasia of mucus-secreting cells and angiogenesis. Such changes may be irreversible, leading to permanent narrowing of the airways [4].

There is much evidence to suggest that adenosine plays a central role in the asthmatic process being the activation of mast cells a key element [5]. In human lung mast cells, adenosine was shown to potentiate mediator release [6]. Similarly, in BALF cells obtained from asthmatic patients after allergen challenge, histamine was shown to be released after exposure to adenosine [7]. The results from these *in vitro* studies are further supported by *in vivo* findings: different studies have shown that inhaled adenosine, or AMP, provoke strong bronchoconstriction in asthmatic patients, but not in healthy subjects (reviewed in [8]), and elevated concentrations of adenosine were observed in BALF of asthmatic patients [9].

The physiological actions of adenosine are mediated by its interaction with specific cell membrane receptors: A_1, A_{2A}, A_{2B}, and A_3. However, the receptor subtypes involved in the adenosine responses in human mast cells are not well characterized. First of all, mast cells from different sources can vary substantially in their biochemical and pharmacological characteristics [10]. In addition, the difficulty in obtaining fresh human mast cell preparations has meant that most researchers have employed surrogates such as cultured human mast cells [11], human mast cell lines [12–14] or mechanically dispersed mast cells from human lungs [6] in their studies. This heterogeneity in mast cell origin has, therefore, hampered progress in this field of research.

There is increasing evidence to suggest that A_{2B} receptors are responsible for the degranulation of mast cells [6,8,12,14–17]. In the human mast cell line HMC-1, adenosine A_{2B} receptors are also linked to the pathogenesis of asthma, partly by the so-called ''enprofylline paradox''. Enprofylline has a potent anti-asthmatic action that has been attributed to its ability to block adenosine A_{2B} receptors, but with low affinity and selectivity [10,12]. It has also been shown that the release of some inflammatory cytokines such as IL-8 or IL-6 from different cells is mediated by the activation of A_{2B} receptors [12,17,18]. The lack of selective adenosine A_{2B} receptor ligands justifies the development of an effective and selective antagonist in order to validate this receptor as therapeutic target in asthma.

The aim of the present study was to profile the potency and selectivity of the new ligand C0036E08 (patent No. WO 03/000694, Example 156) at adenosine receptors. The study revealed C0036E08 as a novel A_{2B} receptor antagonist, which allowed us to investigate the physiological relevance of A_{2B} receptors mediating adenosine-induced histamine release in human mast cells from BALF.

2. Methods and materials

2.1. Reagents

The novel compound C0036E08 belongs to a series of pyrrolopyrimidines bearing a methoxy group at position 3 of the phenoxy ring; the detailed structure and preparation procedure are described in the International application published by the World Intellectual Property Organization (WIPO) with International Publication Number WO 03/000694, Example 156. [^{3}H]DPCPX and [^{3}H]NECA were obtained from Amersham Biosciences (Madrid, Spain). [^{3}H]adenine was purchased from Moravek Biochem ITISA (Madrid, Spain); [^{3}H]ZM241385 and [^{14}C]cAMP from Perkin Elmer (Madrid, Spain). R-PIA, NECA, adenosine deaminase, benzamidine, rolipram, PCA, phenylephrine, acetylcholine, DPCPX, SCH-58261 and MRS 1220 were purchased from Sigma/RBI (Madrid, Spain). Cell culture media and reagents including L-glutamine, and fetal bovine serum were obtained from Sigma (Madrid, Spain), geneticin and zeocin were purchased from Invitrogen (Barcelona, Spain). Protein Assay Kit II was from Bio-Rad, (Hercules, CA, USA). UNIVERSOL™ scintillation cocktail was purchased from MP Biochemicals, Inc. (Irvine, CA, USA).

2.2. Characterization of the pharmacological profile of the novel compound C0036E08

2.2.1. Cell culture

Human embryonic kidney (HEK293) cells stably expressing human adenosine A_{2B} receptors (HEK-A_{2B}) were purchased from Euroscreen (Gosselies, Belgium). Cells were grown as monolayers, at 37 °C in a humified incubator (with 5% CO_2), in nutrient mixture F-12 HAM medium with L-glutamine and $NaHCO_3$, supplemented with 10% non-dialyzed fetal bovine serum, 100 UI/mL penicillin, 100 μg/mL streptomycin and 100 μg/mL geneticin. HeLa cells stably expressing human adenosine A_{2A} (HeLa-A_{2A}) and A_3 receptor (HeLa-A_3) were provided by Dr. Mengod (CSIC-IDIBAPS) and were grown as monolayers, at 37 °C in a humified incubator (with 5% CO_2), in Dulbecco's modified Eagle's medium—high glucose without L-glutamine, supplemented with 10% non-dialyzed fetal bovine serum, 2 mM L-glutamine and 300 μg/mL zeocin. Membrane preparations from these cells were used in binding assays.

Chinese hamster ovary (CHO) cells stably expressing human adenosine A_{2A} receptors (CHO-A_{2A}) and human adenosine A_{2B} receptors (CHO-A_{2B}) prepared at the BioFarma Research Group, were grown at 37 °C in a humified incubator (with 5% CO_2), in Dulbecco's modified Eagle's Nutrient mixture F-12 Ham medium with pyridoxine, $NaHCO_3$, phenol red and L-glutamine, supplemented with 10% non-dialyzed fetal bovine serum and 600 μg/mL geneticin. These cells were used in cAMP assays.

2.2.2. *Binding assays*

2.2.2.1. Adenosine A_1 receptor. Adenosine A_1 receptor competition binding experiments were carried out in membranes from rat cortex as previously described [19]. On the day of assay, membranes were defrosted and re-suspended in buffer 50 mM Tris–HCl, pH 7.4. Each reaction tube, prepared in triplicate, contained 20 μg of membrane protein, 0.5 nM [^{3}H]DPCPX and compound C0036E08 at different concentrations. Non-specific binding was determined in the presence of 10 μM R-PIA. The reaction mixture was incubated at 25 °C for 120 min, after which samples were rapidly vacuum filtered through Whatman GF/C glass filters pre-treated with 0.5% polyethylenimine, in a Brandel cell Harvester (Gaithersburg, MD, USA). The filters were then placed in vials with 4 mL UNIVERSOL™ scintillation cocktail and radioactivity was measured in a beta counter (Beckman LS-6000LL).

2.2.2.2. Adenosine A_{2A} receptor. Adenosine A_{2A} receptor competition binding experiments were carried out in membranes from HeLa-A_{2A} cells. On the day of assay, membranes were defrosted and re-suspended in incubation buffer 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 10 mM $MgCl_2$ and 2 UI/mL adenosine deaminase. Each reaction well of a GF/C multiscreen plate (Millipore, Madrid, Spain), prepared in triplicate, contained 8 μg of protein, 3 nM [^{3}H]ZM241385 and compound C0036E08 at different concentrations. Non-specific binding was determined in the presence of 50 μM NECA. The reaction mixture was incubated at 25 °C for 30 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain).

2.2.2.3. Adenosine A_{2B} receptor. Adenosine A_{2B} receptor competition binding experiments were carried out in membranes from HEK-A_{2B} cells prepared following the supplier's protocol. On the day of assay, membranes were defrosted and re-suspended in buffer: 50 mM Tris–HCl, pH 6.5, 1 mM EDTA, 5 mM $MgCl_2$, 100 μg/mL bacitracine, 2 UI/mL adenosine deaminase. Each reaction well, prepared in triplicate, contained 40 μg of protein, 25 nM [^{3}H]DPCPX and compound C0036E08 at different concentrations. Non-specific-binding was determined in the presence of 1 mM NECA. The reaction mixture was incubated at 25 °C for 30 min in polystyrene plates, after which samples were transferred to a GF/C multiscreen plate (Millipore, Madrid, Spain) and filtered and measured as for A_{2A} receptors.

2.2.2.4. Adenosine A_3 receptor. Adenosine A_3 receptor competition binding experiments were carried out in membranes from HeLa-A_3 cells obtained as previously described [20]. On the day of assay, membranes were defrosted and re-suspended in buffer 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 2 UI/mL adenosine deaminase. Each reaction well, prepared in triplicate, contained 100 μg of protein, 30 nM [^{3}H]NECA and compound C0036E08 at different concentrations. Non-specific binding was determined in the presence of 100 μM R-PIA. The reaction mixture was incubated at 25 °C for 180 min, after which samples were filtered and measured as for A_{2A} receptors.

The protein concentration in all cases was determined with Protein Assay Kit II, based on the Bradford method.

2.2.3. *Functional experiments*

2.2.3.1. Cyclic AMP assays. The adenylyl cyclase activity mediated by A_{2A} and A_{2B} receptors was evaluated in CHO cells stably expressing the receptors, by measuring the levels of cAMP accumulated in response to increasing concentrations of NECA in the absence or presence of compound C0036E08. Briefly, cells grown in 12 well plates with growth medium containing dialyzed fetal bovine serum, were washed twice with DMEM F-12 nutrient mixture medium containing 25 mM HEPES pH 7.4 and 30 μM of the phosphodiesterase inhibitor rolipram (incubation buffer) and then pre-incubated, in 1 mL/well of incubation buffer containing 3 μCi/mL of [^{3}H]adenine, at 37 °C for 2 h in 5% CO_2. The cells were then washed with incubation buffer and different concentrations (0.1 nM–1 mM) of the agonist NECA in the absence or presence of antagonist were added to each well. The incubation was continued for 15 min and then terminated by the addition of 100 μL of PCA with 0.011 mCi/well [^{14}C]cAMP, and plates were placed on ice for 30 min. The amount of intracellular cAMP accumulated was measured by anion exchange chromatography (Dowex-Alumina) with [^{14}C]cAMP as internal standard. Radioactivity was measured in a beta counter (Beckman LS-6000LL).

2.2.3.2. Isolated organ bath studies. Antagonism of C0036E08 at A_{2A} receptors was assayed using thoracic aorta from male (300–350 g) Sprague Dawley rats as previously described [21]. Before addition of the ligands, the tissue strips were equilibrated for 1 h under a 2 g tension load. Isometric force changes were recorded with a Grass FT03C transducer and a Grass 7D polygraph. Following the equilibration period, the rings were sensitized by the addition of 0.1 μM phenylephrine, and 10 μM acetylcholine was then added to test for the presence of endothelium. Equilibration periods (60 min) were then alternated with the construction of relaxating NECA concentration–response curves over 0.1 μM phenylephrine-induced contractions. A control curve (NECA alone) was followed by test curves with NECA in the presence of compound C0036E08, which was added to the bath solution 20 min before the end of the equilibration period.

Antagonism of C0036E08 at A_{2B} receptors was assayed using endothelium denuded thoracic aorta from male (300–350 g) guinea pigs as previously described [22], following a procedure analogous to that described above for the A_{2A} receptor study in rat aorta. Relaxating NECA concentration–response curves were performed over 4 μM phenylephrine-induced contractions.

2.2.4. Expression of results

2.2.4.1. *Binding studies*. The −log of inhibition constant (pK_i) of each compound was calculated by the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{(1 + [L]/K_D)}$$

where IC_{50} is the concentration of compound that displaces the binding of radioligand by 50%, [*L*] is the free concentration of radioligand and K_D is the dissociation constant of each radioligand. IC_{50} values were obtained by fitting the data with non-linear regression, with Prism 2.1 software (GraphPad, San Diego, CA).

2.2.4.2. *Functional experiments*. NECA concentration–response curves in cAMP assays and isolated organ bath studies were fitted to the following equation with Prism 2.1 (Graph Pad, San Diego, CA) and Kaleidagraph software (Synergy Software, Reading, PA), respectively:

$$E = E_{max} \frac{[A]^s}{(EC_{50}^s + [A]^s)}$$

where E_{max}, [*A*], and *s* represent the maximum response, agonist concentration, and curve slope, respectively. EC_{50} is the concentration of agonist that produces 50% of the maximal response. The EC_{50} values are given as means ± S.E.M. (standard error of the mean). The antagonist potency was expressed as pK_B (−log of the dissociation constant, K_B), calculated for one concentration of antagonist following the equation:

$$K_B = \frac{[B]}{((EC'_{50}/EC_{50}) - 1)}$$

where EC'_{50}/EC_{50} is the ratio of concentrations of agonist giving equal responses (50% of the maximal effect) in the presence and in the absence of a given concentration [*B*] of the antagonist, respectively.

2.3. Characterization of adenosine receptors in human mast cells from bronchoalveolar fluid (BALF)

2.3.1. Patients, bronchoscopy and BALF

Patients attending the Pneumology Departments of the Santiago de Compostela Clinical and Provincial Hospital for routine diagnostic bronchoscopy were recruited for the study. The patients were diagnosed with bronchogenic carcinoma or lung fibrosis. All patients underwent a standardized lavage with sterile isotonic saline (3 × 60 mL) inserted under minimum hand pressure. After a short time, the fluid was aspirated into a plastic container and the samples pooled into a polypropylene container. The sample fluid was immediately placed on ice and transferred to the laboratory.

A cell concentrate containing mast cells was obtained from human BALF, as described by Forsythe [23]. An aliquot of the pooled sample was removed for total mast cell counting. Differential cell counts were made by means of the glass coverslip method and staining with Toluidine blue (0.5% in 80% formaldehyde, 20% acetic acid) for 30 min.

2.3.2. Functional studies in human mast cells

2.3.2.1. *Measurement of histamine release in human mast cells*. Human mast cells from BALF (10,000 cells per test tube) were pre-warmed at 37 °C for 5 min and histamine release was measured in Umbreit buffer (1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 22.85 mM $NaHCO_3$, 5.94 mM KCl, 1 mM $CaCl_2$, 119 mM NaCl and 0.1% glucose), at pH 7.0. Histamine release from mast cells was initiated by incubating the cells with the calcium ionophore A23187 at the indicated concentrations, for 5 min at 37 °C. Secretion was elicited by adding the adenosine agonist NECA at the indicated concentrations, for 10 min at 37 °C. The reactions were stopped on ice for 10 min. Cells were then centrifuged at 700 × *g* for 10 min at 4 °C and the supernatants were collected. Histamine release into the supernatants was determined with RIA Kit (Pharmacia) and expressed as a percentage of the total cellular histamine content. The spontaneous release of histamine (i.e. that occurring in the absence of any stimulus) was not subtracted.

2.3.2.2. *Functional characterization of adenosine receptors in human mast cells*. Histamine released in the presence of adenosine antagonists selective for the different adenosine receptor subtypes was determined. Human mast cells from BALF (10,000 cells per test tube) were pre-warmed at 37 °C for 5 min and histamine release was initiated with the calcium ionophore A23187 (0.7 μM). After 2 min, different adenosine antagonists (DPCPX, SCH-58261, C0036E08 or MRS 1220, for A_1, A_{2A}, A_{2B} and A_3 receptors, respectively were added at increasing concentrations (10 pM–1 μM for C0036E08 and 10 pM–0.1 μM for all the other antagonists). After 3 min, secretion was elicited by adding 1 μM NECA for 15 min at 37 °C. The reactions were stopped on ice for 10 min, the cells were then centrifuged at 700 × *g* for 10 min at 4 °C and the supernatants collected.

The direct effect of compound C0036E08 on the histamine release induced by the calcium ionophore at concentrations of 0.7 and 0.9 μM, was assayed by initiating histamine release with the calcium ionophore A23187 and adding different concentrations (10 pM–0.1 μM) of compound C0036E08 2 min later. After 15 min at 37 °C the reactions were stopped on ice for 10 min and the cells were then centrifuged at 700 × *g* for 10 min at 4 °C and the supernatants collected.

3. Results

3.1. Characterization of C0036E08 as an antagonist of adenosine A_{2B} receptors

The affinity of the novel compound C0036E08 (see Fig. 1) for adenosine A_1, A_{2A}, A_{2B}, and A_3 receptor subtypes was determined in radioligand binding assays, with membranes from rat cortex, HeLa-A_{2A}, HEK-A_{2B}, or HeLa-A_3 cells, respectively.

Representative C0036E08 competition curves for radioligand binding of human adenosine A_1, A_{2A}, A_{2B}, and A_3 receptor subtypes are shown in Fig. 2. The compound showed higher affinity for human adenosine A_{2B} receptors (pK_i 8.46) than for rat A_1 and human A_{2A} and A_3 receptors (pK_i 7.49, 5.31, and 4.97, respectively). Hence, C0036E08 displayed high selectivity over

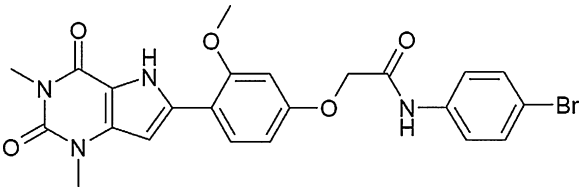

Fig. 1 – Structure of compound C0036E08 (N-(4-bromophenyl)-2-[4-(1,3-dimethyl-2,4-dioxo-2,3,4,5-tetrahydro-1*H*-pyrrolo[3,2-*d*]pyrimidin-6-yl)-3-methoxyphenoxy]acetamide.

A_{2A} and A_3 receptors (K_i ratios *vs* A_{2B} receptor 1412 and 3090, respectively) and moderate selectivity over A_1 receptors (K_i ratio 9) (Table 1, Fig. 2).

A_{2A} and A_{2B} receptors stimulate cAMP production [24] and have a relaxant effect on the contractions induced by phenylephrine, both at A_{2A} receptors in rat aorta [21,25], and at A_{2B} receptors in guinea pig aorta [22]. We studied the antagonist activity of C0036E08 on NECA-induced cAMP accumulation mediated by human A_{2A} and A_{2B} receptors in cell lines and on the relaxant response elicited by NECA in rat and guinea pig aorta (see Fig. 3 and Table 1). A pK_B value of 6.30 was obtained for C0036E08 inhibiting the adenosine A_{2A} receptor-mediated cAMP accumulation induced by NECA in CHO-A_{2A} cells (Fig. 3A), whereas a pK_B value of 8.16 was obtained in a parallel assay for the inhibition of adenosine A_{2B} receptor-mediated cAMP accumulation in CHO-A_{2B} cells (Fig. 3B).

Similarly, in studies measuring the relaxation response mediated by adenosine A_{2A} and A_{2B} receptors in animal tissues, compound C0036E08 was found to attenuate to some extent the relaxation responses via adenosine A_{2A} receptors in rat aorta (Fig. 3C): at 10^{-7} M, the compound showed a slight rightward shift of the control curve, which due to the small magnitude of the effect (dose-ratio between antagonist and control curves lower than two-fold) allowed us only to estimate the pK_B value to be less than 7 (apparent $pK_B = 5.9$). C0036E08 produced a rightward shift of the relaxation–response curves mediated by A_{2B} receptors in endothelium-denuded rings from guinea pig aorta (pK_B 8.3) (Fig. 3D).

The data (summarized in Table 1) demonstrate the selectivity and potency of the novel compound C0036E08 as an A_{2B} receptor antagonist and, therefore, suggest that this molecule is a useful tool for further study of A_{2B} receptor function.

3.2. *Characterization of A_{2B} receptor-mediated histamine release in human mast cells from BALF*

Histamine release was induced by 0.7 μM calcium ionophore A23187 and potentiated by NECA in a concentration-dependent manner ($pEC_{50} = 7.60 \pm 0.05$) in human mast cells from BALF (Fig. 4). In order to evaluate which adenosine receptors were involved in this response to NECA, we studied the effects of different adenosine receptor antagonists on the histamine release induced by 1 μM NECA in human mast cells from BALF. The A_1 receptor antagonist DPCPX, at concentrations selective for this receptor subtype (10 pM–0.1 μM) did not have any

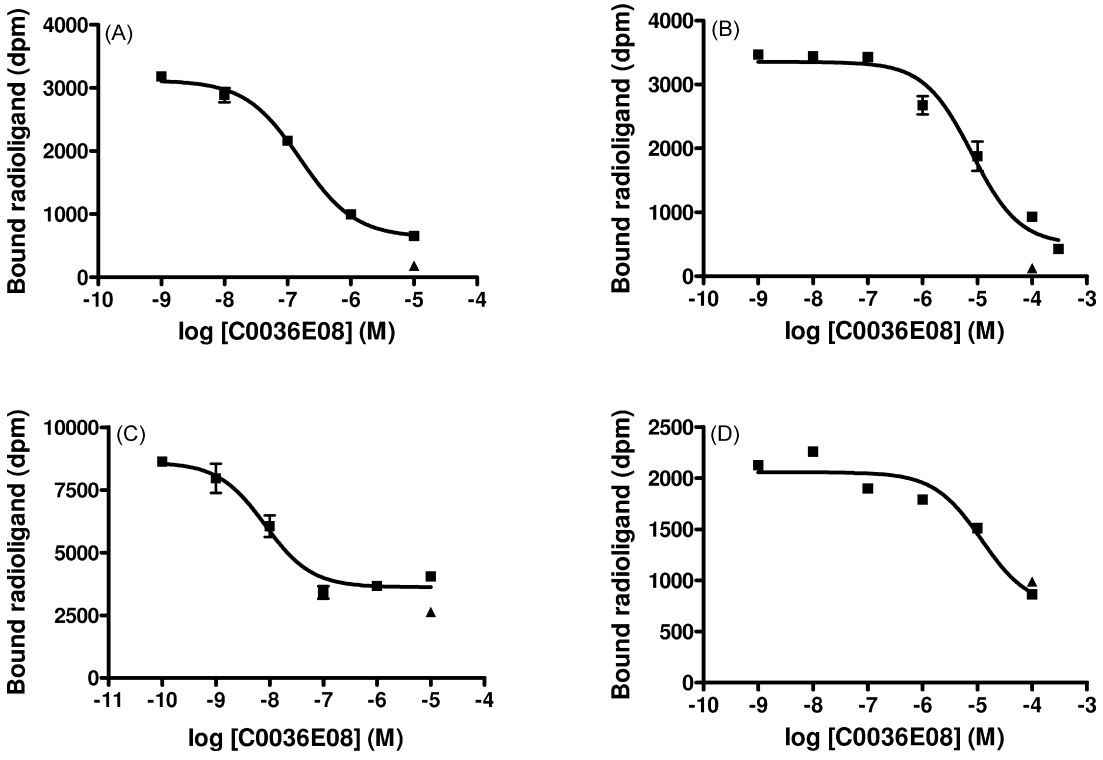

Fig. 2 – Competition of compound C0036E08 (■) for binding of (A) [^{3}H]DPCPX to adenosine A_1 receptors, (B) [^{3}H]ZM241385 to adenosine A_{2A} receptors, (C) [^{3}H]DPCPX to adenosine A_{2B} receptors, and (D) [^{3}H]NECA to adenosine A_3 receptors. (▲) Non-specific binding defined as described in Section 2. Each panel shows the mean ± S.E.M. of a representative experiment out of three independent assays.

Table 1 – Selectivity of C0036E08 for different adenosine receptor subtypes

Adenosine receptors	A_1	A_{2A}	A_{2B}	A_3
Binding (pK_i) (K_i, nM)	7.49 ± 0.53 (32 ± 4.9)	5.31 ± 0.13 (4897 ± 36.7)	8.46 ± 0.51 (3.47 ± 0.2)	4.97 ± 0.19 (10715 ± 20.7)
cAMP (pK_B)	Nd	6.30 ± 0.61	8.16 ± 0.71	Nd
Functional aorta (pK_B)	Nd	<7[a]	8.3 ± 0.9[b]	Nd

Values are expressed as means ± S.E.M., n = 3–5.
Nd: not determined.
[a] Rat aorta.
[b] Guinea pig aorta.

significant effect on the release of histamine evoked by 1 μM NECA (Fig. 5A). A similar lack of effect was observed for SCH-58261 (A_{2A} receptor antagonist) and MRS 1220 (A_3 receptor antagonist) at concentrations selective for each receptor subtype respectively (data not shown). However, C0036E08 induced a complete concentration-dependent inhibition of 1 μM NECA-potentiated histamine release (pEC_{50} = 7.91 ± 0.04) (Fig. 5B). The latter value is consistent with the affinity and functional potency of C0036E08 at A_{2B} receptors measured by binding, cAMP accumulation and guinea pig aorta relaxation assays (pK_i 8.46, pK_B 8.16, and pK_B 8.3, respectively). Furthermore, control experiments performed with 0.7 and 0.9 μM A23187 (minimal and maximal histamine release for this ionophore, respectively) in the absence of NECA confirmed that C0036E08 (10 pM–0.1 μM) does not inhibit histamine release in a non-specific manner (Fig. 5C and D). The effect of C0036E08 may therefore be readily considered specific for the inhibition of the histamine release induced by NECA. Consistently with these functional results, the expression of A_{2B} receptors in the mast cell preparation from human BALF was confirmed by RT-PCR and Western blot studies (Supplementary Figure 1). While the impossibility to obtain a fully purified mast cell preparation from BALF samples might compromise the attribution of the detected A_{2B} mRNA and protein signals to mast cells and not to other possible cell types such as macrophages, together these data support that the adenosine receptor-dependent release of histamine in human mast cell preparations from BALF is an A_{2B}-regulated process.

4. Discussion

Mast cells play an important role in the initiation and amplification of the allergic-inflammatory pathology of

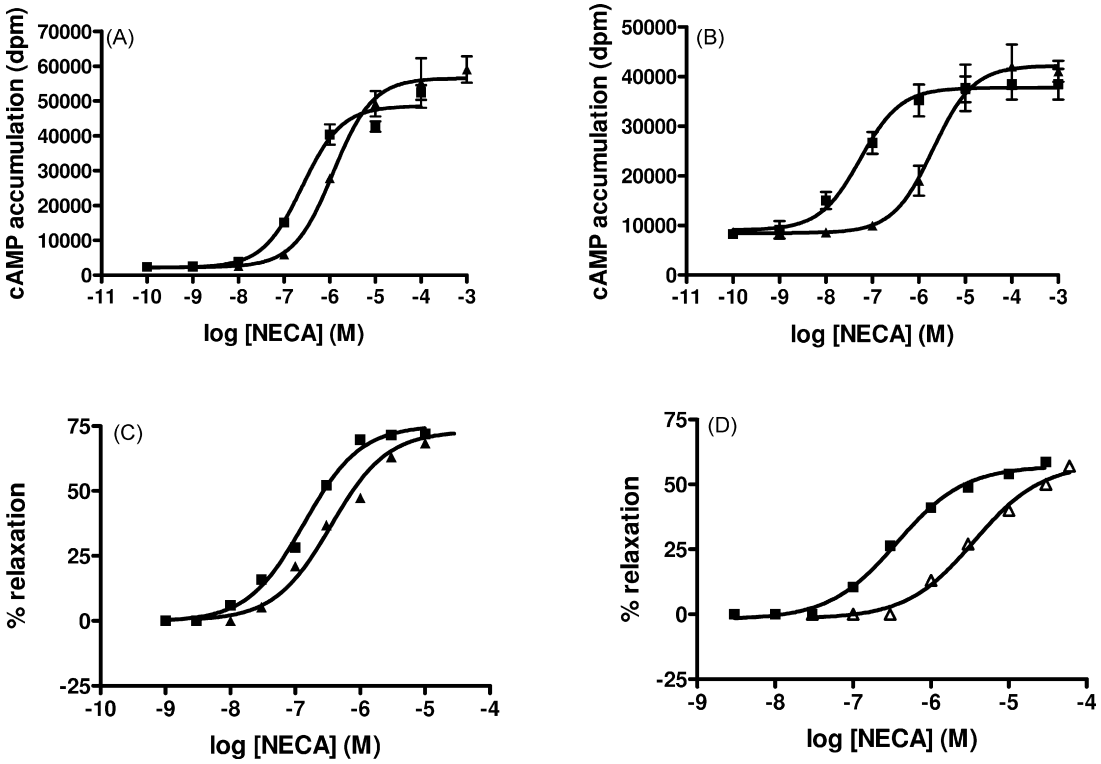

Fig. 3 – Functional antagonist effects of C0036E08 on A_{2A} and A_{2B} receptor-mediated responses. cAMP accumulation (A and B) or relaxation responses (C and D) induced by NECA were measured in (A) CHO-A_{2A} cells, (B) CHO-A_{2B} cells, (C) rat aorta, and (D) guinea pig aorta. Concentration–response curves for NECA were performed in the absence (■) or in the presence of 0.1 μM C0036E08 (▲) or 1 μM C0036E08 (△). Each panel shows the mean ± S.E.M. of a representative experiment out of three independent assays.

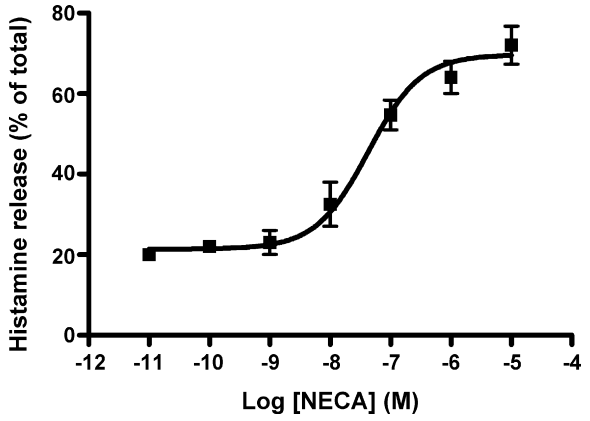

Fig. 4 – NECA-induced histamine release in human BALF. Concentration–response curve for NECA (■) potentiating the histamine release induced by 0.7 μM A23187 in human mast cells from BALF. Histamine release was expressed as the percentage of the total cellular histamine content. The spontaneous release of histamine in the absence of any stimulus was around 10% and it was not subtracted. A23187 (0.7 μM) alone induced a histamine release of 15–20%. The panel shows the mean ± S.E.M. of three independent assays.

asthma through the release of active mediators, and it has also been shown that inhaled adenosine provokes bronchoconstriction in asthmatic patients but not in non-asthmatic subjects. The adenosine pathway in asthma appears to be preferentially mediated by mast cell activation and release of mast cell mediators in BALF, a process that can be blocked by inhibitors of mast cell activation. While the expression of adenosine receptor subtypes might differ amongst mast cells from different sources, mast cells obtained from BALF have been suggested to constitute a good model for the testing of novel, adenosine receptor-targeted therapies for asthma [23], and we used this model in the present work for the testing of a new chemical tool targeting A_{2B} receptors.

Increasing evidence from studies in human mast cells suggests that the adenosine A_{2B} receptor subtype is involved in degranulation of mast cells and, therefore, in the pathogenesis of asthma [5]. However, the above-mentioned differences between mast cells and cell lines, together with the lack of pharmacological tools specific for A_{2B} receptors, represent a challenge to researchers in the field.

To respect to A_{2B} receptor selective tools developed until now, enprofylline represents the first example of a xanthine derivative, and it has shown a potent anti-asthmatic effect attributed to its ability to inhibit degranulation through blockage of adenosine A_{2B} receptors yet with low affinity

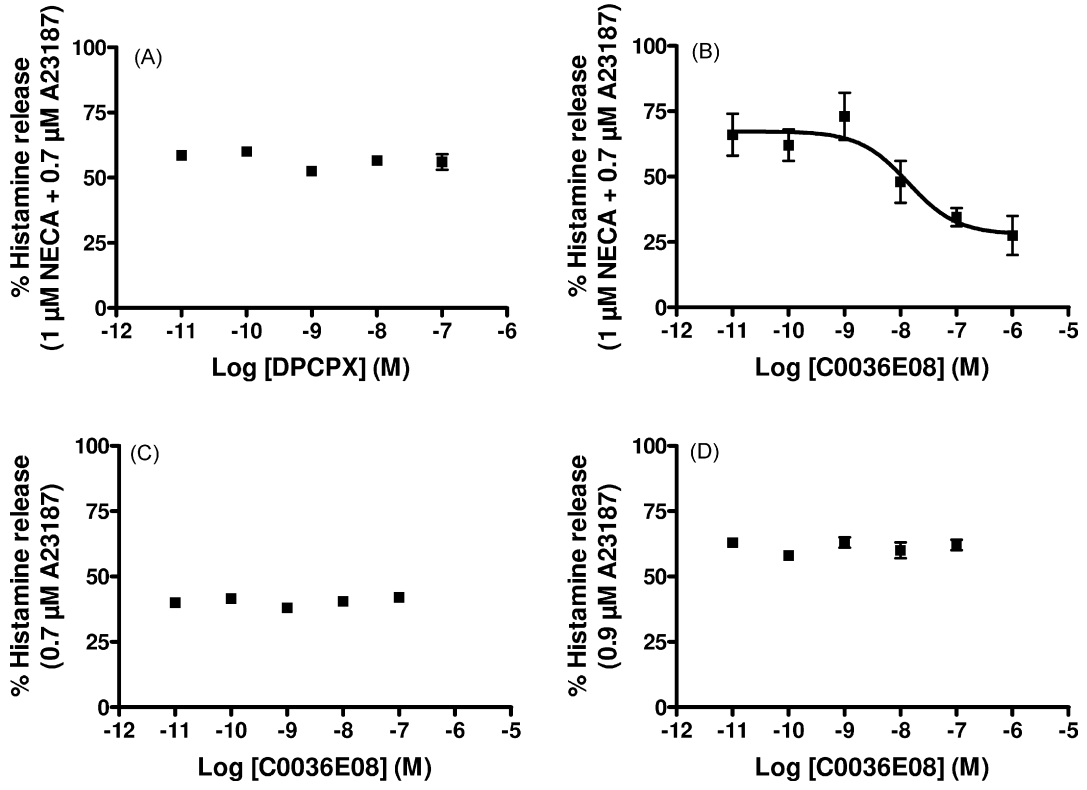

Fig. 5 – C0036E08 inhibited NECA-induced histamine release in human BALF. (A and B) Concentration–response curves (■) for: (A) DPCPX (10 pM–0.1 μM) (A_1-selective antagonist), (B) C0036E08 (10 pM–1 μM) (A_{2B}-selective antagonist) on the histamine release induced by 0.7 μM A23187 + 1 μM NECA in human mast cells from BALF. (C and D) Control experiments showing the absence of unspecific effects of C0036E08 (10 pM–0.1 μM) on the histamine release induced by (C) 0.7 μM or (D) 0.9 μM A23187. Histamine release was expressed as the percentage of the total cellular histamine content. The spontaneous release of histamine in the absence of any stimulus was around 10% and it was not subtracted. The panels show the mean ± S.E.M. of four independent assays.

and selectivity (pK_i 5.20 *vs* pK_i 4.49, 4.35 and 3.80 at A_1, A_{2A}, and A_3 adenosine receptor subtypes respectively, [10,12] and references therein). The development of selective antagonists for adenosine A_{2B} receptors from methylated xanthine chemical structures with multiple substitutions of the parent heterocycle, in particular C(8)-substituted heterocycles, has been one of the most commonly used approaches in targeting A_{2B} receptors. In fact, this strategy led to the identification of several new selective antagonists for A_{2B} receptors such as IPDX (3-isobutyl-8-pyrrolidinoxanthine), reported to show high selectivity although low affinity for A_{2B} receptors (pK_i 6.3 *vs* 4.6, 4.4 and 4.3 at A_1, A_{2A} and A_3 receptors, respectively) and able to inhibit NECA-induced interleukin secretion in HMC-1 cells [17].

Other prototypic adenosine receptor antagonists including the xanthine DPCPX and non-xanthine compounds such as ZM241385 and CGS15943, have a reasonably high affinity for adenosine A_{2B} receptors (pK_i = 6.9, 7.5 and 7.2, respectively) but again they are not selective over other adenosine receptor subtypes [26,27]. Lastly, MRS 1754 (pK_i 8.7 *vs* 6.4, 6.3, 6.2 at A_1, A_{2A} and A_3 receptors, respectively) and OSIP339391 (pK_i 9.30 *vs* 7.43, 6.48, 6.34 at A_1, A_{2A} and A_3 receptors, respectively) are the more selective A_{2B} antagonists [28–30].

The chemical structure described in the present study, C0036E08, is a deazaxanthine, isosteric with xanthine, but it has an electronic character different to other xanthine derivatives, as shown by its lower basicity and its photophysical properties [31]. C0036E08 has the same pharmacophore as MRS 1754, but the main differences between these two compounds are the replacement of the imidazole group of MRS 1754 by a pyrrole in C0036E08, the dimethyl substitution at the pyrimidinone moiety and the presence of the methoxy group, all of which may play a role in changing the conformation of the molecule. This can be specially relevant taking into account that xanthine derivatives have shown limited drug-like characteristics (i.e. low aqueous solubility) and they have been associated with non-specific side effects (i.e. cytochrome inhibition), which have led to the withdrawal of these compounds from clinical trials. Hence, new xanthine derivatives with slightly different chemical structures (e.g. deazaxanthines, such as C0036E08) may contribute to progress in the development of more drug-like and safer A_{2B}-selective antagonists.

C0036E08 showed high affinity (pK_i 8.46) and significant selectivity for A_{2B} adenosine receptors (9 times, 1412 times and 3090 times higher than for A_1, A_{2A} and A_3 human adenosine receptors, respectively; $P < 0.001$; Student's *t*-test) (Table 1). Furthermore, C0036E08 selectively antagonized NECA-induced relaxation in guinea pig aorta (pK_B 8.3), yet it was only a weak antagonist of the A_{2A}-mediated relaxation of rat aorta by this agonist ($pK_B < 7$). The compound showed similar selectivity antagonizing the cAMP response mediated by human A_{2B} (pK_B 8.16) and A_{2A} (pK_B 6.30) receptors in cell lines, discarding putative inter-species differences at the binding or functional level. Furthermore, C0036E08 exhibited higher selectivity for A_{2B} over A_{2A} and A_3 receptors than the prototypic A_{2B} antagonist MRS 1754. The data reveal C0036E08 as a valuable tool for characterizing A_{2B} receptors in native systems.

Adenosine-induced bronchoconstriction is observed in asthmatic patients but not in non-asthmatic subjects, and it is also well known that the non-selective adenosine receptor agonist NECA indirectly potentiates mast cell degranulation and histamine release induced by other agents (e.g. calcium-mobilizing compounds, contractile agonists) [12]. Accordingly to this, in the present study NECA did not activate mast cells directly but potentiated the histamine release induced by the calcium ionophore A23187 in human mast cells freshly obtained from BALF. The mast cell preparation obtained from BALF following our protocol might contain other cell types such as macrophages. However, the assumption that the observed effects on histamine release experiments involved mast cells seems reasonable in the light of the findings that adenosine can directly activate the human mast cell line HMC-1 [12], as well as reports in animal models of direct activation of mast cells by adenosine *in vivo*. Furthermore, RT-PCR and Western blot studies indicated the expression of A_{2B} receptors in our human mast cell preparations from BALF (Supplementary Figure 1), accordingly to a possible pathophysiological role of A_{2B} receptors in human mast cells and in agreement with the reported expression of adenosine A_{2A}, A_{2B} and A_3 receptors in mast cells from different sources and species [5,13,32–34]. However, the existence of A_{2B} receptors in macrophages might prevent the attribution of the detected A_{2B} expression signals from these experiments to mast cells origin exclusively.

In our functional experiments in human mast cells, we demonstrated that the selective A_{2B} antagonist C0036E08 was able to fully block NECA-mediated histamine release in a concentration-dependent manner, without affecting the histamine release induced by the ionophore A23187. Furthermore, the lack of effect of the selective A_1 antagonist DPCPX (10 pM–0.1 μM) on the NECA-mediated histamine release in these cells discarded any eventual contribution of blockade of A_1 receptors (the adenosine receptor for which C0036E08 shows the highest affinity after A_{2B}) to the inhibition of the histamine release by C0036E08 in human mast cells from BALF. DPCPX was tested at a concentration range (10 pM–0.1 μM) selective for A_1 receptors *vs* A_{2B} receptors. At these concentrations, any effect of this antagonist on histamine release through A_{2B} receptors is expected to be very small (approximately 20% inhibition), which would result barely detectable using our methodology. In other hand, recent descriptions of increased expression of adenosine A_1 receptor in bronchial epithelium and smooth muscle from asthmatic patients [35] suggest that the combined antagonism of A_{2B} receptors in mast cells and A_1 bronchial receptors might be a potential dual mechanistic approach for the treatment of asthma. From this point of view, a certain anti-A_1 component in the pharmacological profile of A_{2B} antagonists might be considered of therapeutic interest.

All together is consistent with the presence of functional adenosine A_{2B} receptors regulating histamine release in human mast cells from BALF and compound C0036E08, which has been profiled as a novel potent and selective A_{2B} receptor antagonist, was able to fully block NECA-induced histamine release in these cells. In conclusion, this work validates compound C0036E08 as a valuable tool in the characterization of human A_{2B} receptor-dependent processes, providing the basis for the development of new selective A_{2B}-targeted drugs of potential interest as anti-asthmatic agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.07.011.

Conflict of interest

None.

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