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REVIEW

Adenosine receptors and asthma in humans

CN Wilson

Chief Scientific Officer, Endacea, Inc., Research Triangle Park, NC, USA

According to an executive summary of the GINA dissemination committee report, it is now estimated that approximately 300 million people (5% of the global population or 1 in 20 persons) have asthma. Despite the scientific progress made over the past several decades toward improving our understanding of the pathophysiology of asthma, there is still a great need for improved therapies, particularly oral therapies that enhance patient compliance and that target new mechanisms of action. Adenosine is an important signalling molecule in human asthma. By acting on extracellular G-protein-coupled ARs on a number of different cell types important in the pathophysiology of human asthma, adenosine affects bronchial reactivity, inflammation and airway remodelling. Four AR subtypes (A_1, A_{2a}, A_{2b} and A_3) have been cloned in humans, are expressed in the lung, and are all targets for drug development for human asthma. This review summarizes what is known about these AR subtypes and their function in human asthma as well as the pros and cons of therapeutic approaches to these AR targets. A number of molecules with high affinity and high selectivity for the human AR subtypes have entered clinical trials or are poised to enter clinical trials as anti-asthma treatments. With the availability of these molecules for testing in humans, the function of ARs in human asthma, as well as the safety and efficacy of approaches to the different AR targets, can now be determined.
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Keywords: adenosine; ARs; asthma; A_1 AR; A_{2a} AR; A_{2b} AR; A_3 AR

Abbreviations: ADA, adenosine deaminase; AMP, adenosine monophosphate; APCs, antigen presenting cells; AR, adenosine receptor; BAL, bronchoalveolar lavage; CFTR, cystic fibrosis transmembrane conductance regulator; CPA, N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; FEV_1, forced expiratory volume in one second; GINA, Global Initiative for Asthma; HBECs, human bronchial epithelial cells; HBSMCs, human bronchial smooth muscle cells; ICSs, inhaled corticosteroids; IP(3), inositol trisphosphate; KO, knockout; LABAs, long acting beta 2 agonists; LAMAs, long acting muscarinic antagonists; LTRAs, leukotriene receptor antagonists; MCP, monocyte chemotactic protein; NECA, 5′-*N*-ethylcarboxamidoadenosine; PC_{20} for AMP, provocative concentration (PC) of AMP required to reduce FEV_1 by 20%; PDE-IV, phosphodiesterase-IV; RT-PCR, reverse transcription-polymerase chain reaction

Introduction

Unmet medical need for new drugs that improve patient compliance and prevent and treat airway remodelling

According to an executive summary of the GINA dissemination committee report, it is now estimated that approximately 300 million people (5% of the global population or 1 in 20 persons) have asthma (Masoli *et al*., 2004). Moreover, according to the World Health Organization, 255 000 people died of asthma in 2005 and asthma is the most common chronic disease among children. Despite the scientific progress made over the past several decades toward improving our understanding of the pathophysiology of asthma, there is still a great need for improved therapies, particularly oral therapies that enhance patient compliance. A high percentage of patients with asthma (20%) are not controlled with currently available therapies and a high percentage of patients (>60%) are not compliant with inhaled treatments for asthma (Jones *et al*., 2003; Bender and Rand, 2004; Sin *et al*., 2004). Patient non-adherence to treatments for chronic diseases accounts largely for an increase in health-care costs and reduced patient quality of life (Bender and Rand, 2004). Asthma control is thus strongly dependent on patient compliance. Despite the recent recommendations for the use of ICSs as the first-line therapy for asthma, there is poor patient compliance (<40%) with all inhaled treatments (Jones *et al*., 2003). Moreover, despite its low efficacy as an anti-asthma drug, sales for the widely prescribed oral drug for asthma, Singulair, have reached a $4 billion run rate in 2007 suggesting that patients prefer oral drugs.

Emerging therapies for asthma include PDE-IV inhibitors, LTRAs, anti-cytokines, syk kinase inhibitors, anti-IgE

Correspondence: Dr CN Wilson, Chief Scientific Officer, Endacea, Inc., PO Box 12076 (Mail), 2 Davis Drive (Courier), Research Triangle Park, NC 27709-2076, USA.
E-mail: cwilson@endacea.nctda.org
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treatment, new ICSs, LABAs, LAMAs, combinations of ICSs and LABAs or LAMAs or a combination of LABAs and LAMAs. The diversity of these pharmacological approaches to asthma treatment stems not only from the clinical heterogeneity of asthma syndromes, but also from the efficacy of specific therapies in differing severities of disease, as well as from whether management of acute exacerbations or long-term maintenance therapy is the primary goal. PDE-IV inhibitors are only modestly effective and because of their low therapeutic index, if approved, may be limited to use in mild to moderate asthmatics. Moreover, because of their low efficacy, especially in more severe asthmatics, either PDE-IV inhibitors or LTRAs cannot be used as monotherapies, as supplementation with acute rescue drugs, that is, short-acting inhaled β-2 agonists, antimuscarinic agents and/or ICSs would be required to treat acute exacerbations. Furthermore, in doses that are clinically safe and well tolerated, it is unclear that approved bronchodilator and anti-inflammatory asthmatic treatments, for example, LTRAs, ICSs, combinations of ICSs and LABAs, LAMAs or a combination of LABAs and LAMAs prevent or reverse structural airway remodeling, involving mucus gland hyperplasia, subepithelial fibrosis, hypertrophy of bronchial smooth muscle and angiogenesis (Jarjour and Kelly, 2002; Cohn *et al*., 2004). These structural changes of airway remodelling lead to a progressive loss of lung function associated with fixed airway narrowing in patients with asthma (Silva *et al*., 2004). Moreover, emerging anti-asthma and anti-inflammatory treatments in doses that are clinically safe and well tolerated may also not prevent airway remodelling and the progressive loss of lung function in human asthmatics. Additional limitations to the development of biological products, including ease of administration, shelf life, lot-to-lot variations, manufacturing quality assurance, costs, and potential for anaphylaxis and side effects, such as thrombocytopenia, may prevent the development and acceptance of these products as anti-asthma drugs. Finally, higher doses of ICSs than that are recommended by the current guidelines as well as anti-cytokine therapies may produce immune suppression and increase the risks for opportunistic infections, as well as in the case of ICSs' suppression of the hypophyseal–pituitary–adrenal axis. Therefore, there is an unmet medical need for a new class of safe, effective oral drugs with a novel mechanism of action in asthma that targets specific pathophysiological mechanisms to prevent the progressive loss of lung function associated with airway remodelling and, in so doing, treats the underlying disease and not just the symptoms.

Adenosine and human asthma

Adenosine is a primordial endogenous nucleoside-signalling molecule, which, by acting on extracellular ARs, produces a number of physiological and pathophysiological effects in the human body, including bronchoconstriction and inflammation. It is now recognized that adenosine is an important signalling molecule in human asthma and has an important function in both the acute bronchoconstrictor and airway inflammatory responses in humans:

- Adenosine levels are increased in the BAL fluid and exhaled breath condensate of patients with allergic asthma and in the plasma of patients with exercise-induced asthma (Driver *et al*., 1993; Huszar *et al*., 2002; Vizi *et al*., 2002).
- The sensitivity of airways to adenosine and AMP, which is metabolized locally by the ectonucleotidase, 5′ nucleotidase, to adenosine, more closely reflects an inflammatory process and the phenotype for allergic asthma than does sensitivity to other known bronchoprovocative agents, for example, methacholine and histamine (de Meer *et al*., 2002; Holgate, 2002; Spicuzza *et al*., 2003).
- Adenosine induces hyperresponsiveness in the airways of asthmatics, but not in normal patients, both *in vivo* following inhalation and *in vitro* in small airways (Cushley *et al*., 1983; Dahlen *et al*., 1983; Bjorck *et al*., 1992).
- At therapeutic plasma levels less than those required to inhibit PDE, both theophylline, a non-selective AR antagonist and bamiphylline, a selective A_1 AR antagonist (which does not bind to human A_{2b} or A_3 ARs), improve lung function and symptoms in humans with asthma (Foutillan *et al*., 1983; Abbrachio and Cattabeni, 1987; Ginesu *et al*., 1987; Catena *et al*., 1988; Morandini, 1988; Crescioli *et al*., 1991; Gaspardone *et al*., 1993; Barnes, 2003; Obiefuna *et al*., 2005).

ARs and human asthma

Adenosine elicits hyperreactive airway responses in humans with allergic asthma by acting on extracellular ARs. Four G-protein-coupled AR subtypes (A_1, A_{2a}, A_{2b} and A_3) have been cloned in humans, are expressed in the lung, and are all targets for drug development for human asthma (Marquardt, 1997; Polosa, 2002; Rorke and Holgate, 2002; Fredholm, 2003; Livingston *et al*., 2004).

A_1 ARs and human asthma

Under normal physiological conditions, A_1 ARs are quiescent; however, A_1 ARs are upregulated in conditions of stress, such as ischaemia, and in conditions of inflammation, typified by the inflammatory airway involvement in human asthmatics (Lai *et al*., 2005; Rogachev *et al*., 2006; Brown *et al*., 2008). With the use of immunohistochemistry and an antibody with high affinity and high selectivity for the human A_1 AR, the distribution and quantification of A_1 AR expression were determined in specimens obtained from bronchial biopsies in AMP-sensitive, atopic, steroid-naive, non-smoking asthmatics with mild asthma ($n=11$) and in healthy, non-smoking normal individuals ($n=7$). In these studies, A_1 ARs are upregulated in airway epithelium and bronchial smooth muscle in human asthmatics (Brown *et al*., 2008). In the Figures (1–2), immunostaining with the A_1 AR antibody is shown in brown against a blue background from Mayer's haematoxylin counterstaining. In asthmatics, there is a strong positive staining for A_1 ARs in bronchial epithelium and moderate–strong staining in bronchial smooth muscle (Figure 1). As opposed to asthmatics, the immunostaining with the A_1 AR antibody is weak in the

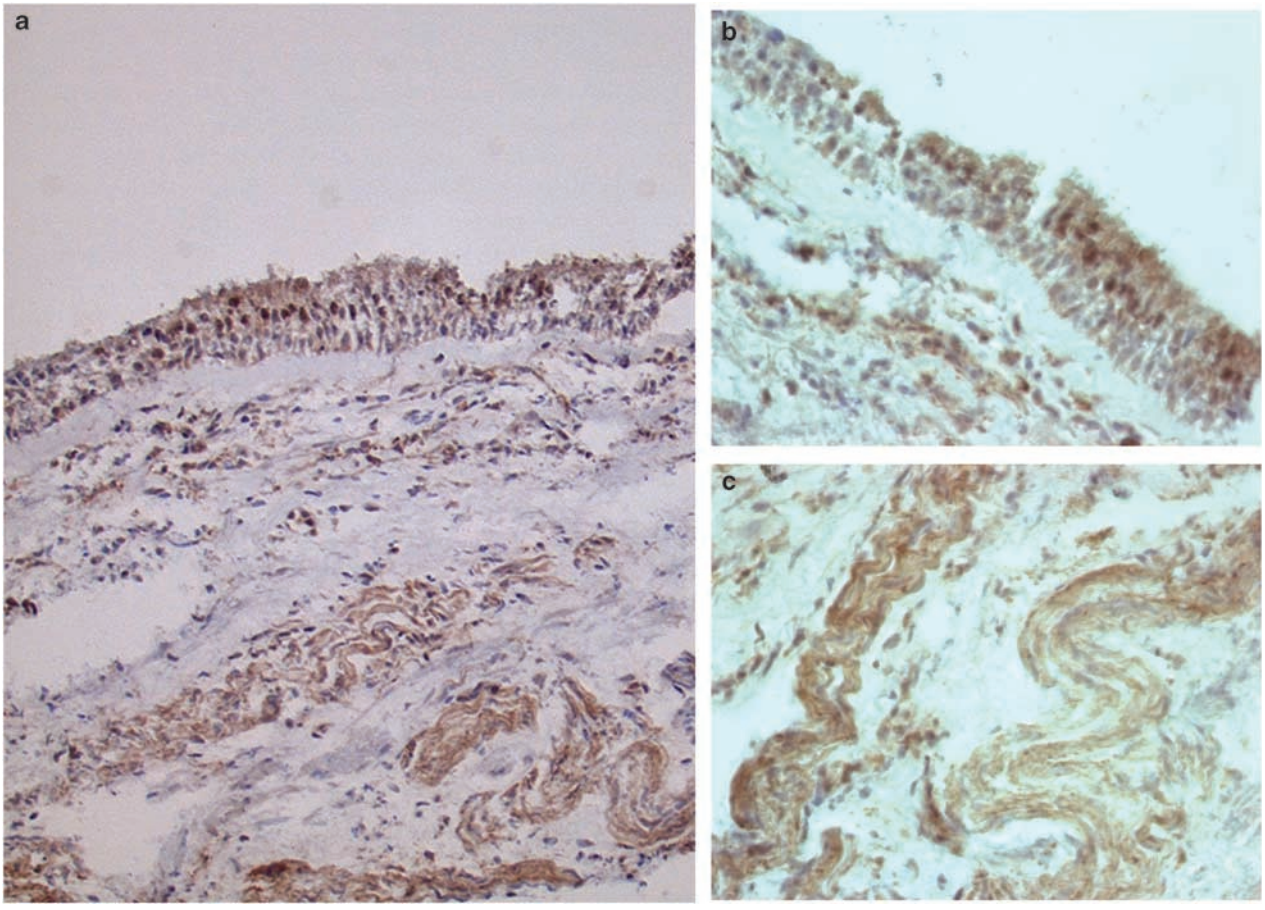

Figure 1 Representative photomicrographs showing positive immunostaining of asthmatic bronchial biopsy sections with an A_1 receptor antibody (**a**), with high expression of the A_1 receptor on the epithelium (**b**) and smooth muscle (**c**), shown at higher magnification. Positive immunostaining appears brown against a blue background as a result of the Mayer's haematoxylin counterstain. Scale bars = 25 μm. Reprinted with permission from Brown *et al.* (2008).

epithelium and very weak in the bronchial smooth muscle of normal volunteers (Figure 2).

In these studies, with the use of image analysis, quantification of staining demonstrates a significant increase in expression of the A_1 AR in airway epithelium and bronchial smooth muscle in asthmatics versus normal healthy controls ($P<0.01$) (Figure 3). These data are confirmed by another laboratory in a preliminary study in a small number of patients, asthmatics and normal controls. In lung samples obtained from asthmatics ($n=3$) and normal donors ($n=3$), with the use of RT-PCR, gene expression for A_1 ARs is increased by approximately 200% in bronchial tissue from small airways of asthmatics versus those of normal controls (unpublished data, S Jamal Mustafa and Ahmed Nadeem, West Virginia University). In these studies, the expression of A_{2a} ARs is decreased and there is little to no change in the expression of A_{2b} ARs and A_3 ARs in bronchial tissue from small airways of asthmatics versus those of normal controls. The results from these studies with the use of RT-PCR to determine the expression of ARs in small airways in human asthmatics versus normal donors were confirmed with the use of western blots, with the exception of the A_{2b} AR, which was not tested in western blots.

The A_1 AR couples through a pertussis toxin-sensitive inhibitory G protein to a number of effector systems, including adenylate cyclase, phospholipase A_2, phospholipase C, potassium channels, calcium channels and guanylate cyclase (van Galen *et al.*, 1992; Akbar *et al.*, 1994; Olah and Stiles, 2000; Fredholm *et al.*, 2001). A_1 ARs have been described on a number of different human cell types that are important in the pathophysiology of asthma, including APCs, human airway epithelial and bronchial smooth muscle cells, lymphocytes, mast cells, neutrophils, monocytes, macrophages, fibroblasts and endothelial cells (Cronstein *et al.*, 1990, 1992; Salmon and Cronstein, 1990; Marone *et al.*, 1992; Salmon *et al.*, 1993; Ahmed *et al.*, 1995; Peachell *et al.*, 1998; Forsythe *et al.*, 1999; Murakami *et al.*, 2001; Panther *et al.*, 2001; Wilson and Batra, 2002; McNamara *et al.*, 2004; Ethier and Madison, 2006; Clark *et al.*, 2007; Brown *et al.*, 2008). Activation of A_1 ARs on these different cell types induces the release of mediators and cytokines that lead to airway hyperreactivity, inflammation and airway remodelling. Activation of A_1 ARs on human asthmatic bronchial tissue produces bronchoconstriction (Bjorck *et al.*, 1992). Moreover, in studies with HBSMCs, activation of A_1 ARs rapidly mobilizes intracellular calcium

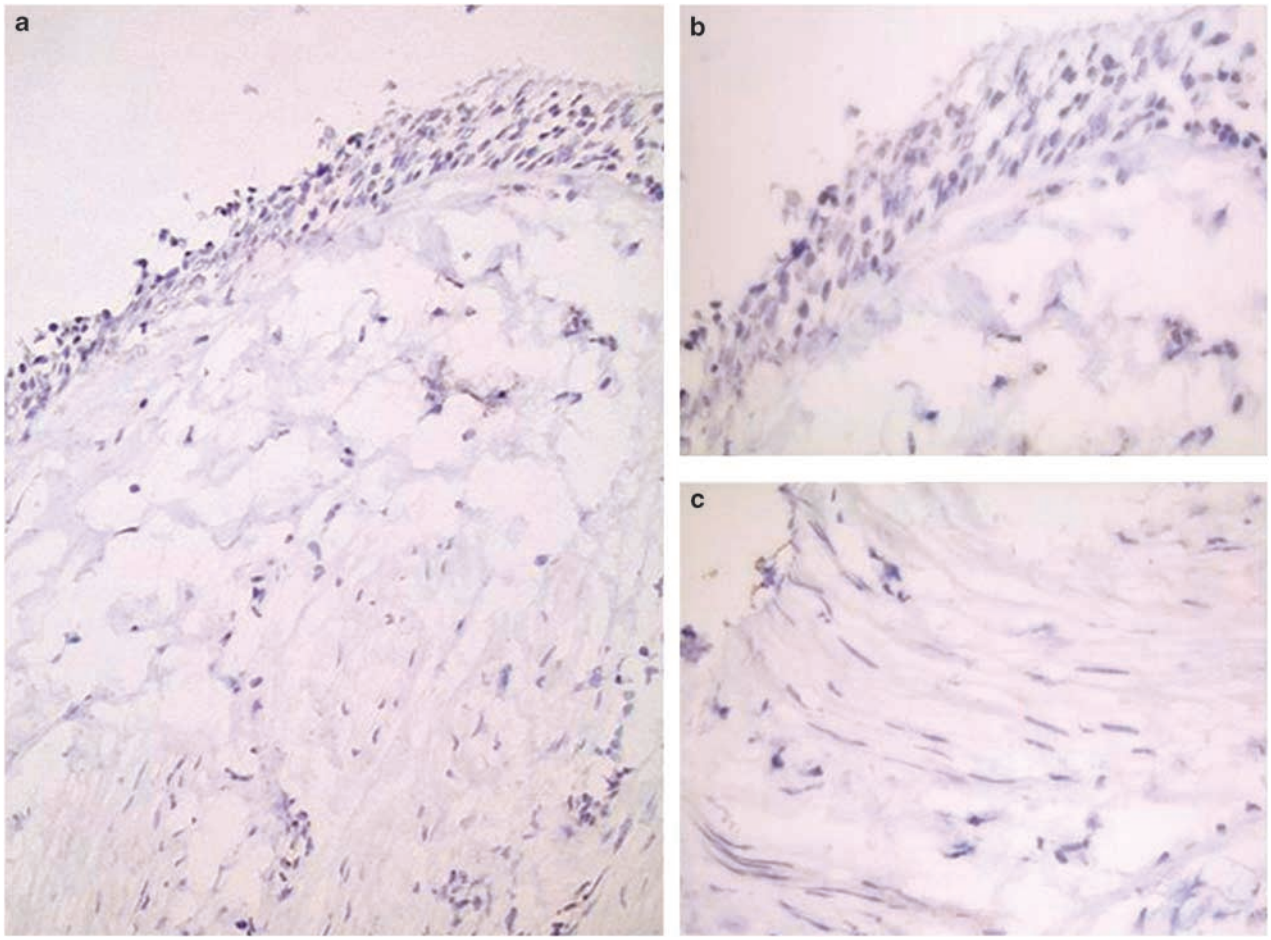

Figure 2 Representative photomicrographs showing positive immunostaining of healthy bronchial biopsy sections with an A_1 receptor antibody (**a**), with weak expression of the A_1 receptor on the epithelium (**b**) and virtually no positive immunostaining of smooth muscle (**c**), shown at higher magnification. Positive immunostaining appears brown against a blue background as a result of the Mayer's haematoxylin counterstain. Scale bars = 25 μm. Reprinted with permission from Brown *et al.* (2008).

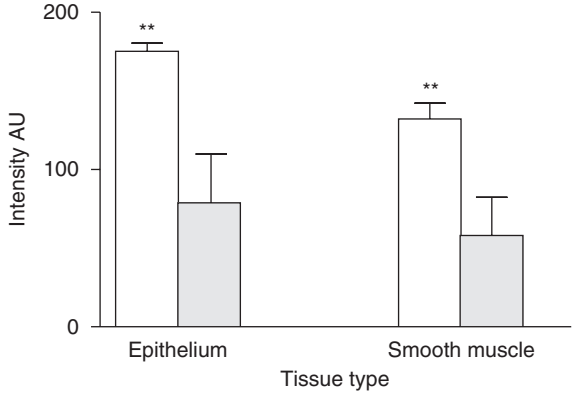

Figure 3 Image analysis of asthmatic (open columns) and healthy (filled columns) bronchial biopsy sections immunostained with an A_1 receptor antibody. The intensity of positive staining specifically on the epithelium and smooth muscle areas of bronchial biopsy sections from 11 asthmatic volunteers and seven healthy individuals (one section per volunteer) was quantified using a Zeiss Vision KS400 software, from four different views of both epithelium and smooth muscle at a magnification of ×1000. Data are presented as mean ± s.e.m. $^{**}P<0.01$ versus healthy individuals. Reprinted with permission from Brown *et al.* (2008).

through a mechanism that is dependent on a pertussis toxin-sensitive G protein and IP(3) (Ethier and Madison, 2006). In these studies, in HBSMCs, the effects of a selective A_1 AR agonist, CPA, and the non-selective AR agonist, NECA, on calcium responses were blocked by pertussis toxin and by a selective A_1 AR antagonist, DPCPX, suggesting that NECA was acting on an A_1 AR in HBSMCs to increase calcium release. These A_1 AR-mediated effects of NECA on calcium signalling were dependent on intracellular calcium, IP(3) and phospholipase C signalling.

Activation of A_1 ARs on other human cell types, including airway epithelial cells, mast cells, neutrophils, cultured monocytes, macrophages and endothelial cells induces the release of substances, which cause bronchoconstriction, pro-inflammatory cellular effects, airway oedema and mucus secretion, all of which lead to airflow obstruction in asthma (Cronstein *et al.*, 1990, 1992; Salmon and Cronstein, 1990; Salmon *et al.*, 1993; Peachell *et al.*, 1998; Forsythe *et al.*, 1999; Wilson and Batra, 2002; McNamara *et al.*, 2004; Ethier and Madison, 2006; Clark *et al.*, 2007). On human neutrophils, activation of A_1 ARs induces neutrophil chemotaxis, adherence of activated neutrophils to endothelial cells, and Fcγ

receptor-mediated phagocytosis and superoxide anion generation (Cronstein *et al*., 1990, 1992; Salmon and Cronstein, 1990). In cultured human monocytes, the effect of adenosine on high-affinity A_1 ARs is pro-inflammatory and enhances Fcγ receptor-mediated phagocytosis and induces the release of vascular endothelial growth factor (Salmon *et al*., 1993; Clark *et al*., 2007). Moreover, it was reported earlier that the activation of A_1 ARs on human pulmonary artery endothelial cells induces the release of thromboxane and IL-6 (Wilson and Batra, 2002). Furthermore, it was reported that upregulation of the *MUC 2* gene on human airway epithelial cells is increased in the presence of adenosine (McNamara *et al*., 2004). This effect of adenosine seems to be mediated by the activation of the A_1 AR and signalling transduction pathways through a Ca^{2+}-activated Cl^- channel that leads to the activation of the epidermal growth factor receptor and upregulation of the *MUC 2* gene in human airway epithelial cells (McNamara *et al*., 2004).

In contrast to these pro-inflammatory effects of A_1 ARs, in human cells, studies with genetically modified mice suggest that the A_1 AR may produce anti-inflammatory effects (Sun *et al*., 2005). The functional role of the A_1 AR was evaluated in ADA KO mice and in ADA/A_1 AR double KO mice. ADA is a ubiquitous enzyme responsible for the inactivation of adenosine. ADA-deficient mice exhibited increased levels of adenosine and increased levels of the A_1 AR transcript, which was most predominant in alveolar macrophages (Sun *et al*., 2005). These ADA-deficient animals developed pulmonary inflammation, characterized by an increase in macrophages, eosinophils, fibrosis, mucus metaplasia and airway hyperreactivity, a phenotype similar to that of allergic asthma. Pulmonary inflammation was exacerbated in mice lacking both ADA and the A_1 AR (Sun *et al*., 2005). Animals with ADA deficiency die between 18 and 21 days and those lacking both ADA and the A_1 AR die between 15 and 16 days. Interestingly, adenosine levels in the lungs of ADA A_1 AR double KO mice were increased approximately by 200% higher than those in ADA KO mice (Sun *et al*., 2005). Moreover, the predominant cell type in ADA A_1 AR double KO mice is the macrophage, with the eosinophils and lymphocytes accounting for less than 2% of the total cells in BAL fluid. The relevance of this ADA A_1 AR double KO mouse model to that of allergic human asthma, where eosinophils and lymphocytes are predominant cell types in the airways, should be interpreted with caution. Moreover, differences in the phenotypes of genetically modified animals are very complex and not yet completely understood. For example, the A_1 AR interacts with ADA (Ciruela *et al*., 1996; Saura *et al*., 1996). Perhaps, A_1 AR KO mice are functionally ADA-deficient accounting for the higher levels of adenosine in ADA A_1 AR double KO mice. Furthermore, in ADA KO mice, high adenosine levels produce lung injury despite a significant increase in the expression of the A_1 AR (Sun *et al*., 2005). In addition, in genetically manipulated animals, compensatory expression of other proteins that alter the phenotypes of cells and organs may play a more important role in the organ injuries seen in these models than is understood at this time.

In summary, the expression of A_1 ARs is increased in the epithelium and airway smooth muscle of airways of human asthmatics. In human airway tissue and HBSMCs, activation of A_1 ARs produces effects that cause airway hyperresponsiveness. On human airway epithelial cells, activation of A_1 ARs causes an increase in expression of the *MUC 2* gene responsible for mucus hypersecretion. Moreover, activation of A_1 ARs on a number of different human cells produces pro-inflammatory effects. Taken together, these effects of A_1 ARs in humans suggest that the A_1 AR is an important target in human asthma. This is further supported by the findings that suggest a methylxanthine, bamiphylline, produces its anti-asthma effects in humans by blocking A_1 ARs (Obiefuna *et al*., 2005). On account of these effects of A_1 ARs and because of the safety and efficacy of bamiphylline as an anti-asthma drug, L-97-1 (Endacea, Inc., Research Triangle Park, NC, USA), is in late preclinical development as a once daily, oral treatment for human asthma. L-97-1 is a water-soluble small-molecule A_1 AR antagonist with high affinity and high selectivity for the human A_1 AR (Obiefuna *et al*., 2005). Compared with bamiphylline, L-97-1 has higher affinity and selectivity for the human A_1 AR (Obiefuna *et al*., 2005). In an animal model of allergic asthma, L-97-1 blocks allergic airway responses, airway hyperresponsiveness to histamine, and airway inflammation (Obiefuna *et al*., 2005; Nadeem *et al*., 2006). Moreover, a number of A_1 AR antagonists have been in clinical trials for a number of different indications and, as a class, seem to be safe and well tolerated in humans (Foutillan *et al*., 1983; Catena *et al*., 1988; Morandini, 1988; Gaspardone *et al*., 1993; Barrett, 1996; Bertolet *et al*., 1996; Gottlieb *et al*., 2002; Doggrell, 2005; Dittrich *et al*., 2007; Greenberg *et al*., 2007; Givertz *et al*., 2007). Three A_1 AR antagonists, Adentri (BG 9928) (Biogen Idec, Cambridge, MA, USA), SLV320 (Solvay Pharmaceuticals SA, Brussels, Belgium) and rolofylline (KW 3902) (Merck & Co. Inc., Whitehouse Station, NJ, USA) are currently in phase II (SLV320) and phase III (Adentri, BG 9928 and KW 3902, rolofylline) clinical trials for the treatment of congestive heart failure with renal impairment (59–62; http://clinicaltrials.gov; Biogen press release 21 August 2008). Following completion of phase II clinical trials for BG 9928 and KW 3902 no safety concerns were reported (Doggrell, 2005; Givertz *et al*., 2007; Greenberg *et al*., 2007). There are no reports for clinical trials for SLV 320 to date. According to the Drug Safety Unit Officer of Chiesi, bamiphylline has a very low incidence of undesired effects (<1 per 100 000 patients exposed, rare cases of headache and gastralgia) (personal communication).

Another molecule that targets the A_1 AR has been in clinical trials as an anti-asthma drug. In a small clinical trial conducted by EpiGenesis Pharmaceuticals (Cranbury, NJ, USA) in patients with asthma a single dose of EPI-2010, an anti-sense ('knock-out') compound for the human A_1 AR, reduced the need for bronchodilator drugs to control asthma symptoms concomitant with a reduction in symptom scores in the patients treated with EPI-2010 (Ball *et al*., 2003). This effect was statistically and clinically significant and lasted for 1 week following a single dose. However, because of disappointing results in a phase II clinical trial, EPI-2010 was discontinued from clinical testing (Langley *et al*., 2005). In this phase II clinical trial, 146 patients with persistent airway obstruction (FEV_1 74.5% predicted, $\geqslant$12% reversibility) and currently receiving ICSs were administered EPI

2010 (1, 3, or 9 mg) through a nebulizer once or twice weekly for 29 days. There was no significant change in the FEV_1 after 29 days of treatment compared with baseline. EPI-2010 showed no additional therapeutic effect in patients currently receiving ICSs. Patients with a stable FEV_1 of 74.5% predicted have mild/moderate asthma, depending on the frequency of symptoms and magnitude of variability in the peak expiratory flow rate. In patients with mild/moderate asthma treated with ICSs, the FEV_1 may be 90–100% of the predicted value when measured between exacerbations and without provocation. Thus, the FEV_1 is not a sensitive measure of asthma severity *per se*, vis-a-vis acute changes in airway function reflected by peak expiratory flow rate variability in ICS-treated patients with mild/moderate asthma. Moreover, in this study, because of safety concerns with the use of antisenses as therapeutics in humans, it is possible that the doses of EPI-2010 were subtherapeutic.

A_{2a} ARs and human asthma

In addition to A_1 ARs, A_{2a}, A_{2b} and A_3 ARs have been described on a number of human cell types that are important in the pathophysiology of human asthma and are targets for development of anti-asthma drugs (Marquardt, 1997; Polosa, 2002; Rorke and Holgate, 2002; Fredholm, 2003; Livingston *et al*., 2004). A_{2a} ARs are coupled through Gs to adenylate cyclase, which, following activation, results in an increase in intracellular cAMP. As activation of A_{2a} ARs increases intracellular cAMP similar to other agents that increase intracellular cAMP, for example, PDE-IV inhibitors, β-2 agonists or dibutyryl cAMP, A_{2a} AR agonists may represent a new class of anti-asthma drugs that produce bronchodilation and anti-inflammatory effects. Activation of A_{2a} ARs produces anti-inflammatory effects in human neutrophils, monocytes/macrophages and lymphocytes (Cronstein *et al*., 1990, 1992; Salmon and Cronstein, 1990; Marone *et al*., 1992; Fredholm *et al*., 2001; Sullivan, 2003; Hasko and Cronstein, 2004). On activated human neutrophils, stimulation of A_{2a} ARs inhibits Fcγ receptor-mediated phagocytosis and superoxide anion production, elastase release, inhibition of upregulation of β-2 integrins and shedding of L-selectin, as well as neutrophil adherence to endothelial cells (Cronstein *et al*., 1990, 1992; Salmon and Cronstein, 1990; Marone *et al*., 1992; Fredholm *et al*., 2001; Sullivan, 2003; Hasko and Cronstein, 2004). Activation of A_{2a} ARs on monocytes/macrophages inhibits cytokine release and in human basophils, activation of A_{2a} ARs inhibits antigen-induced histamine and leukotriene release (Hasko and Cronstein, 2004). Moreover, activation of A_{2a} ARs on endothelial cells prevents IL-8 and IL-6 release and reduces endothelial permeability (Hasko and Cronstein, 2004). An A_{2a} AR agonist, CGS 21680, decreases airway inflammation in an allergic animal model of asthma (Fozard *et al*., 2002). In human asthmatics, however, an A_{2a} AR agonist, GW 328267X, did not block allergen-induced early and late asthmatic responses or inflammation (Luijk *et al*., 2008). In non-smoking, atopic asthmatics ($n = 15$), who underwent allergen challenge following treatment for 1 week with GW 328267X (25 μg, twice daily) in a double-blind, placebo- and fluticasone (250 μg)-controlled study, this A_{2a} AR agonist did not protect against allergen-induced early or late asthmatic reaction, or the accompanying inflammatory response as measured by sputum total cell counts, number of EG2+ cells, and the concentration of IL-8 and eosinophil cationic protein.

Activation of A_{2a} AR induced rises in intracellular cAMP also produces vasodilation and reflex tachycardia, a normal sympathetic nervous system response to a reduction in blood pressure (Sullivan, 2003). These cardiovascular side effects of hypotension and reflex tachycardia reduce the therapeutic index for A_{2a} AR agonists as drug candidates in humans even following their administration as an inhalational treatment to avoid these systemic side effects (Luijk *et al*., 2008). Although GW 328267X was administered as an inhalational treatment, GlaxoSmithKline discontinued the clinical development of this A_{2a} AR agonist because of its low therapeutic index. As mentioned above, at a low dose GW 328267X did not produce efficacy in asthmatics in a phase Ib clinical trial (Luijk *et al*., 2008). At higher doses, this A_{2a} AR agonist produced cardiovascular side effects (decrease in blood pressure and an increase in heart rate). Moreover, because activation of A_{2a} ARs produces neovascularization (angiogenesis), an A_{2a} AR agonist, MRE-0094 is in phase II clinical trials as a treatment for wound healing in diabetic foot ulcers (Aderis Pharmaceuticals, Hopkinton, MA, USA) (Montesinos *et al*., 1997, 2006; Cronstein, 2006). However, because angiogenesis is a cardinal feature of the airway remodelling of human asthma, this effect of A_{2a} AR agonists may limit their clinical development as anti-asthma drugs.

The chronic use of A_{2a} AR agonists as anti-asthma drugs may lead to tachyphylaxis and immune suppression that would further limit their clinical efficacy and safety. For example, with chronic administration of A_{2a} AR agonists, tachyphylaxis to the bronchodilator and anti-inflammatory effects may occur through the desensitization of Gs-coupled intracellular signalling pathways (Sullivan, 2003). Following chronic administration for 2 weeks, tacyhyphylaxis to the blood pressure-lowering effect of an A_{2a} AR agonist, CGS 21680, prevented its development as an anti-hypertensive agent (Webb *et al*., 1993). Furthermore, A_{2a} AR agonists exert an effect through Gs to stimulate adenylate cyclase in a similar manner to that of LABAs and may be subject to safety concerns similar to those associated with chronic use of LABAs. The Food and Drug Administration has issued a warning that use of LABAs may increase the risk of sudden death in asthmatics (Salpeter *et al*., 2004). Finally, activation of A_{2a} ARs leads to anti-tumour effects and immune suppression (Sullivan, 2003; Ohta *et al*., 2006). By blocking the oxidative and non-oxidative activity of neutrophils, causing functional repression and/or apoptosis of lymphocytes, and inhibiting the release of IL-12, which promotes bacterial clearance in infection, A_{2a} AR agonists may cause immune suppression and predisposition to infection (Sullivan, 2003). Moreover, activation of A_{2a} ARs in an adenosine-rich tumour microenvironment produces inhibition of anti-tumour T cells (Ohta *et al*., 2006). The use of AR antagonists or targeting A_{2a} ARs with siRNA pretreatment of T cells improved inhibition of tumour growth, destruction of metastasis and prevention of neovascularization by anti-tumour T cells. Genetic

deletion of the A_{2a} AR resulted in the rejection of established immunogenic tumours with no rejection in wild-type mice. Despite what should be advantageous effects, that is, bronchodilation and anti-inflammatory effects, the potential side effects of hypotension, tachycardia, tachyphylaxis and immune suppression, as well as the angiogenesis and anti-tumour effects produced by A_{2a} AR agonists, may limit the clinical development of A_{2a} AR agonists as anti-asthma drugs.

A_{2b} ARs and human asthma

A_{2b} ARs are also targets for drug development in human asthma. A_{2b} ARs have been described on human bronchial epithelial and smooth muscle cells, monocytes, endothelial cells and fibroblasts (Feoktistov *et al.*, 2002; Zhong *et al.*, 2004, 2005, 2006; Russo *et al.*, 2006). A_{2b} ARs have been described on HMC-1 cells and activation of A_{2b} ARs on these cells coupled through Gq induces the release of inflammatory mediators, which are important in human asthma (Feoktistov and Biaggioni, 1995; Ryzhov *et al.*, 2004). It is reported that through coupling to Gq and intracellular signalling pathways, including PLC, activation of A_{2b} ARs in this cell line induces the release of cytokines, IL-4, IL-13, IL-8 and IL-1β, which, in turn, induces the release of IgE from B cells (Ryzhov *et al.*, 2004). However, HMC-1 cells are derived from a highly malignant undifferentiated human mastocytoma cancer. The relevance of activation of A_{2b} ARs through Gq coupling in these HMC-1 cells to that in IgE immunologically sensitized mast cells of human asthma is unknown. In the allergic response, antigens bind and cross-link IgE molecules bound to the functional high affinity receptor for IgE, FcεRI, on mast cells to induce degranulation and the release of a broad spectrum of pro-inflammatory mediators. HMC-1 cells do not express FcεRI (Nilsson *et al.*, 1994). Moreover, the presence of A_{2b} ARs on IgE immunologically sensitized human mast cells has not been reported.

In other human cell types that are important in the pathophysiology of human asthma, including airway epithelial and bronchial smooth muscle cells, as well as fibroblasts, A_{2b} ARs are coupled through Gs to adenylate cyclase (Zhong *et al.*, 2004, 2005, 2006). Activation of A_{2b} ARs on HBECs induces the release of IL-19, which, in turn, induces the release of TNF-α from human monocytes, which increases the expression of A_{2b} ARs on HBECs (Zhong *et al.*, 2006). In HBSMCs, activation of A_{2b} ARs coupled to Gs induces the expression and release of IL-6 and MCP-1 (Zhong *et al.*, 2004). IL-6 has an important function in airway remodelling by promoting mucus gland hyperplasia, bronchial smooth muscle hyperplasia and hypertrophy, subepithelial fibrosis and myofibroblast hyperplasia. Moreover, MCP-1, a C-C class chemokine, mediates leukocyte infiltration and activation, T-cell differentiation and airway hyperresponsiveness (Zhong *et al.*, 2004). In human lung fibroblasts, activation of A_{2b} ARs coupled to adenylate cyclase through Gs induces the release of IL-6 and differentiation of fibroblasts to myofibroblasts (Zhong *et al.*, 2005). In these studies, hypoxia increased the expression of A_{2b} ARs on human fibroblasts and enhanced the effect of A_{2b} AR activation on IL-6 release and differentiation of fibroblasts to myofibroblasts. On account of these effects of activation of A_{2b} ARs on human airway epithelial and bronchial smooth muscle cells and fibroblasts to induce the release of cytokines and mediators that may result in airway remodelling, including bronchial smooth muscle hypertrophy, subepithelial fibrosis and differentiation of fibroblasts to myofibroblasts, an A_{2b} AR antagonist, CVT 6883 (CV Therapeutics, Palo Alto, CA, USA), is in phase I clinical trials as an anti-asthma drug. In a mouse model of allergic asthma, CVT 6883 significantly reduced AMP-induced increases in airway resistance, as well as the late allergic response and airway inflammation following allergen challenge (Mustafa *et al.*, 2007). Moreover, in ADA-deficient mice, CVT 6883 decreased pulmonary inflammation, fibrosis and alveolar airspace enlargement and reduced elevations of pro-inflammatory cytokines and chemokines, as well as mediators of fibrosis and airway destruction (Sun *et al.*, 2006). This pro-inflammatory effect of A_{2b} ARs in animals was reported in another study, where NECA-induced increases in IL-6 plasma levels *in vivo* and IL-6 release from mouse peritoneal macrophages *ex vivo* were abrogated in A_{2b} AR KO mice (Ryzhov *et al.*, 2008). Furthermore, in this study, selective A_{2b} AR antagonists, IPDX and MRS 1754, significantly reduced NECA-induced IL-6 release from mouse peritoneal macrophages *ex vivo* in wild-type mice.

As opposed to these reports suggesting that the activation of A_{2b} ARs has an important function in airway reactivity, inflammation and remodelling in allergic asthma, recent reports suggest that the activation of A_{2b} ARs may produce bronchorelaxant and anti-inflammatory effects. In a recent study in guinea pigs, the non-selective AR agonist NECA evoked relaxing responses of isolated tracheal preparations pre-contracted with histamine in normal and sensitized animals, and this effect was reversed by the A_{2b} AR antagonist, MRS 1706 (Breschi *et al.*, 2007). Moreover, *in vitro* desensitization with NECA markedly reduced the relaxing effect of NECA, raising the possibility that higher adenosine levels in the lung might desensitize this receptor to cause bronchorelaxation (Sun *et al.*, 2006). Furthermore, activation of A_{2b} ARs may produce anti-inflammatory effects. In A_{2b} AR KO/reporter gene knock-in mice there was low-grade baseline inflammation, augmented release of pro-inflammatory cytokines, including TNF-α and IL-6, as well as leukocyte adhesion to the vasculature (Yang *et al.*, 2006).

It is possible that the bronchorelaxant and anti-inflammatory effects of A_{2b} ARs described above may be because of an increase in intracellular cAMP levels following activation of A_{2b} ARs. As mentioned earlier, A_{2b} ARs are coupled through Gs and stimulate adenylate cyclase to produce an increase in intracellular cAMP. Also, as noted, it is well known that through its intracellular effects, cAMP produces a non-specific relaxant effect on bronchial and vascular smooth muscle, as well as anti-inflammatory effects in inflammatory cells and decreases in endothelial permeability. Given these effects of intracellular cAMP, it is unclear why an approach to the treatment of asthma would be to block these salutary effects of activation of A_{2b} ARs coupled through Gs to stimulate adenylate cyclase to increase intracellular cAMP. As activation of A_{2b} ARs on endothelial cells reduces endothelial permeability, the use of A_{2b} AR

antagonists may increase endothelial permeability (Lennon *et al*., 1998). Moreover, through coupling to Gs and adenylate cyclase, A_{2b} ARs may have an important function in the control of the CFTR-operated Cl^- channel in human airway epithelial cells (Clancy *et al*., 1999; Huang *et al*., 2001; Rollins *et al*., 2008). As this channel has an important function in airway hydration, the use of A_{2b} AR antagonists may induce a cystic fibrosis-like phenotype associated with an increased viscosity of mucus in humans, and may therefore limit their development as anti-asthma drugs. Any drug that reduces airway hydration and promotes such inspissation of secretions in the airway of an asthmatic with overproduction of mucus would not be safe, as thickened airway secretions can lead to widespread mucus plugging and increased expiratory airflow limitation. On account of this effect of A_{2b} ARs on the Cl^- channel in intestinal epithelial cells, it is suggested that A_{2b} AR antagonists may be developed as anti-diarrhea agents (Strohmeier *et al*., 1995; Kolachala *et al*., 2006). Finally, it was recently reported that a mixed A_{2b}/A_3 AR antagonist, QAF 805 (Novartis International AG, Basel, Switzerland), failed to increase the provocative concentration $(PC)_{20}$ for AMP (concentration of AMP required to reduce the FEV_1 by 20%) versus placebo in 24 AMP-sensitive asthmatics in a placebo-controlled, double-blind, randomized, two-way crossover phase Ib clinical trial (Pascoe *et al*., 2007). Thus, although based on what is reported in animals and human cell lines, it seems that A_{2b} ARs may have an important function in the airway reactivity, inflammation and remodelling of asthma, because of their effect on the CFTR Cl^- channel in human airway epithelial cells and airway hydration, the safety of A_{2b} AR antagonists in human asthmatics remains to be determined.

A_3 ARs and human asthma

The concept of the A_3 AR as a target for development of anti-asthma drugs in humans is somewhat confusing. This confusion is based on reports to date, suggesting that both A_3 AR agonists and A_3 AR antagonists may have potential as anti-asthma agents. The contrasting effects of A_3 ARs are presented in an excellent recent review on the A_3 AR as an 'enigmatic player in cell biology' (Gessi *et al*., 2008). On the basis of earlier reports that activation of A_3 ARs inhibits migration of human eosinophils and produces anti-inflammatory cellular effects in human neutrophils, monocytes and macrophages by inhibiting oxidative burst, degranulation and release of inflammatory cytokines, it has been suggested that A_3 AR agonists may be developed as anti-asthma drugs (Knight *et al*., 1997; Baraldi *et al*., 2000; Fishman and Bar-Yehuda, 2003; Nadeem and Mustafa, 2006). In animals, however, activation of A_3 ARs produces hypotension and bronchospasm (Baraldi *et al*., 2000). Moreover, rapid desensitization follows activation of A_3 ARs; thus, the use of A_3 AR agonists may be associated with tachyphylaxis/tolerance to the beneficial effects of A_3 AR agonists (Fishman and Bar-Yehuda, 2003). Furthermore, because activation of A_3 ARs produces anti-inflammatory effects, the chronic use of A_3 AR agonists may produce immune suppression. On account of the anti-inflammatory and, specifically, the anti-TNF-α effects of activation of A_3 ARs on human monocytes, an A_3 AR agonist, CF 101, is in phase IIb clinical trials for the treatment of rheumatoid arthritis (Can-Fite Biopharma Ltd., Petah-Tikva, Israel). It is reported that CF 101 has an acceptable safety, tolerability profile in humans (van Troostenburg *et al*., 2004). In this report, bronchospasm was not reported as a side effect of CF 101; however, the patients in this study were taking another anti-inflammatory immune suppressant, methotrexate, and CF 101 has not been tested in humans with asthma. Moreover, a second A_3 AR agonist, CF 102, is now in phase I clinical trials for the treatment of liver disorders (Can-Fite Biopharma). The results for tolerability of this A_3 AR agonist in humans are pending completion of these phase I clinical trials.

What about the development of A_3 AR antagonists as anti-asthma drugs? As the activation of A_3 ARs induces the release of preformed mediators from basophils and produces bronchoconstriction, eosinophil migration into airways and mucus hypersecretion in animals, A_3 AR antagonists have been recommended for development as anti-asthma drugs (Fishman and Bar-Yehuda, 2003; Nadeem and Mustafa, 2006). An A_3 AR antagonist, MRS-1220 significantly inhibited 5′-AMP-induced migration of eosinophils and macrophages into the airways of allergen-sensitized guinea pigs (Spruntulis and Broadley, 2001). However, as mentioned above, a mixed A_{2b}/A_3 AR antagonist, QAF 805 (Novartis), failed to increase the PC_{20} for AMP versus placebo in 24 AMP sensitive asthmatics in a placebo-controlled, double-blind, randomized, two-way crossover study phase Ib clinical trial (Pascoe *et al*., 2007).

ARs and human asthma: conclusions

In summary, adenosine is an important signalling molecule in human asthma. Adenosine levels are elevated in the BAL fluid of human asthmatics. By acting on extracellular G-protein-coupled ARs, including A_1, A_{2a}, A_{2b} and A_3 AR subtypes on a number of different human cell types, adenosine affects bronchial reactivity and inflammation, the release of cytokines and mediators, endothelial permeability, fibrosis, angiogenesis and mucus production, all of which are important in the pathophysiology of human asthma. All four G-protein-coupled AR subtypes have been cloned in humans, are expressed in the lung, and are targets for drug development for human asthma. The expression of A_1 ARs is upregulated in the airways of patients with asthma versus normal volunteers. In humans, activation of A_1 ARs on a number of different cell types, including bronchial epithelial and smooth muscle cells, inflammatory cells and endothelial cells, produces bronchoconstriction and inflammation, upregulation of the *MUC 2* gene responsible for mucin hypersecretion in the airways of asthmatics and angiogenesis. On account of these A_1 AR-mediated effects and because, as a class of drugs, A_1 AR antagonists seem to be safe in humans, the A_1 AR is an important target in human asthma and the use of A_1 AR antagonists represents a promising approach to the treatment of human asthma.

On account of their effects on a number of different human cell types involved in airway reactivity, inflammation and airway remodelling, other AR subtypes, A_{2a}, A_{2b}, and A_3 ARs are important targets for anti-asthma drug

Table 1 Therapeutics targeting adenosine receptors

	A_1 AR antagonists	*A_{2a} AR agonists*	*A_{2b} AR antagonists*	*A_3 AR agonists*	*A_3 AR antagonists*
Potential effects in asthmatics	Bronchodilation; anti-inflammatory; inhibition of mucus hypersecretion; block angiogenesis	Bronchodilation; anti-inflammatory	Anti-inflammatory; inhibit airway remodelling	Anti-inflammatory	Bronchodilation; anti-inflammatory; inhibit mucus hyperplasia
Disadvantages	No safety concerns in clinical trials with BG9928; KW3902; no reports for SLV320; no safety concerns in humans with bamiphylline	CV side effects, hypotension, tachycardia; tachyphylaxis; immune suppression	Reduce airway hydration and increase mucus viscosity	Tachyphylaxis; immune suppression	
Latest developments	L-97-1 (preclinical, asthma); EPI-2010 (phase II; D/C, no additional effect with ICSs, asthma); BG 9928 (Adentri, phase III, ADHF); SLV320 (phase II, ADHF); KW3902 (rolofylline, phase III, ADHF); bamifylline (registered, Europe, asthma)	GW328267X (phase II; D/C CV side effects, asthma); UK-432097 (phase II, COPD); ATL 146e (Apadenoson, phase III, D/C, cardiac imaging); ATL 313 (preclinical, sepsis, reperfusion organ injury) MRE 0094 (phase II, wound healing diabetic foot ulcers)	CVT 6883 (phase I, asthma); QAF 805 (phase Ib, asthma)	CF 101 (phase II, rheumatoid arthritis); CF 102 (phase I, hepatitis)	QAF 805 (phase Ib, asthma)
Pharmaceutical company involved in AR drug discovery	Endacea, Inc.; Epigenesis Pharmaceuticals; Biogen Idec; Solvay Pharmaceuticals; Merck	Glaxo Group Ltd.; Pfizer; Adenosine Therapeutics; Aderis Pharmaceuticals	CV Therapeutics; Novartis	Can-Fite Biopharma	Novartis

Abbreviations: AR, adenosine receptor; ADHF, acute decompensated heart failure; COPD, chronic obstructive pulmonary disease; CV, cardiovascular; D/C, discontinued.

development. However, there are pros and cons associated with approaches to these different AR targets. By acting on A_{2a} ARs coupled to Gs and adenylate cyclase to increase intracellular cAMP to produce bronchodilation and anti-inflammatory cellular effects, it is expected that A_{2a} AR agonists may be attractive drug candidates as anti-asthma treatments. Unfortunately, the cardiovascular side effects of A_{2a} AR agonists, that is, a decrease in blood pressure and increase in heart rate, may reduce the therapeutic index and limit the development of these molecules as anti-asthma drugs. By coupling to Gs and adenylate cyclase, activation of A_{2b} ARs on different human cell types induces the release of cytokines and mediators that are important in the pathophysiology of airway remodelling in human asthma. For these reasons, an A_{2b} AR antagonist, CVT 6883, is in a phase I clinical trial for development as an anti-asthma drug (CV Therapeutics). Moreover, based on reports in animals, A_{2b} AR antagonists may reduce airway reactivity and airway inflammation of allergic asthma. However, because activation of A_{2b} ARs on HBECs may have an important function in control of the CFTR Cl^- channel, A_{2b} AR antagonists may reduce airway hydration, thus promoting inspissation of secretions and mucus plugging in patients with asthma and an overproduction of mucus. This effect of A_{2b} AR antagonists on airway hydration and mucus viscosity in asthmatics has not been determined; however, it may limit the safety and efficacy of this class of drugs in patients with asthma. Finally, the A_3 AR as a target for development of anti-asthma drugs is confusing. On the one hand, it is reported that activation of A_3 ARs inhibits the migration of human eosinophils and produces anti-inflammatory effects in human neutrophils and monocytes. For these reasons, A_3 AR agonists have been suggested for the treatment of asthma. In contrast, because activation of A_3 ARs induces inflammatory cell migration and mucus production in animal models of allergic asthma, A_3 AR antagonists have been suggested for the treatment of asthma. As summarized in Table 1, a number of molecules with high affinity and high selectivity for the human AR subtypes have entered clinical trials or are poised to enter clinical trials. With the availability of these molecules for testing in humans, the function of ARs in human asthma, as well as the safety and efficacy of approaches to the different AR targets for novel anti-asthma treatments, can now be determined.

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Conflict of interest

Dr Constance N Wilson is the Founder and Chief Scientific Officer of Endacea, Inc., a biopharmaceutical company in Research Triangle Park, NC that is developing an A_1 AR antagonist, L-97-1, as an oral anti-asthma therapy.

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