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REVIEW

Treating lung inflammation with agonists of the adenosine A_{2A} receptor: promises, problems and potential solutions

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Adenosine A_{2A} receptor agonists may be important regulators of inflammation. Such conclusions have come from studies demonstrating that, (i) adenosine A_{2A} agonists exhibit anti-inflammatory properties *in vitro* and *in vivo*, (ii) selective A_{2A} antagonists enhance inflammation *in vivo* and, (iii) knock outs of this receptor aggravate inflammation in a wide variety of *in vivo* models. Inflammation is a hallmark of asthma and COPD and adenosine has long been suggested to be involved in disease pathology. Two recent publications, however, suggested that an inhaled adenosine A_{2A} receptor agonist (GW328267X) did not affect either the early and late asthmatic response or symptoms associated with allergic rhinitis suggesting that the rationale for treating inflammation with an adenosine A_{2A} receptor agonist may be incorrect. A barrier to fully investigating the role of adenosine A_{2A} receptor agonists as anti-inflammatory agents in the lung is the side effect profile due to systemic exposure, even with inhalation. Unless strategies can be evolved to limit the systemic exposure of inhaled adenosine A_{2A} receptor agonists, the promise of treating lung inflammation with such agents may never be fully explored. Using strategies similar to that devised to improve the therapeutic index of inhaled corticosteroids, UK371,104 was identified as a selective agonist of the adenosine A_{2A} receptor that has a lung focus of pharmacological activity following delivery to the lung in a pre clinical *in vivo* model of lung function. Lung-focussed agents such as UK371,104 may be suitable for assessing the anti-inflammatory potential of inhaled adenosine A_{2A} receptor agonists.
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Abbreviations: ATL146e, 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester; CGS21680, 2-[4-(2-carboxyethyl)phenethylamino]-5′-ethylcarboxamidoadenosine; GW328267, (2R,3R,4S,5R)-2-{6-amino-2-[(1-benzyl-2-hydroxyethyl)amino]-9H-purin-9-yl}-5-(2-ethyl-2H-tetrazol-5-yl)tetrahydrofuran-3,4-diol; UK371,104, *N*-(2,2-diphenylethyl)-2-{[(2-piperidin-1-ylethyl)amino]carbonyl}adenosine

Introduction

Inflammation is a hallmark of diseases such as asthma and chronic obstructive pulmonary disease (COPD). Inhaled corticosteroids and, to a lesser extent, oral leukotriene D4 antagonists have proven efficacious in asthma, although corticosteroids are much less effective in COPD. A continuing focus of research is to discover new and effective non-steroidal anti-inflammatory agents for asthma and COPD (see Barnes, 2006, 2007). This review article will be focussed on discussing the potential broad-spectrum anti-inflammatory potential of inhaled adenosine A_{2A} receptor agonists for asthma and COPD and will not cover other adenosine receptors. For a broader view on the potential role of adenosine and adenosine receptors in the pathology of asthma and COPD, the reader is referred to the following recent reviews (see, van den Berge *et al.*, 2007; Brown *et al.*, 2008).

Adenosine produces a wide range of biological effects by interacting with four cell surface receptors termed A_1, A_{2A}, A_{2B} and A_3. These receptors have been identified by both molecular and pharmacological techniques (Fredholm *et al.*, 2001). Of the four receptors, the adenosine A_{2A} receptor has been strongly linked to of control inflammation (see below). The receptor is very widely distributed and agonism of this receptor produces a wide range of physiological responses such as hypotension, inhibition of platelet aggregation and regulation of neurotransmitter release (Fredholm *et al.*, 2001). Indeed A_{2A} receptor antagonists have been proposed to have a potential therapeutic benefit in CNS diseases such as Parkinsons (Schwarzschild, 2007). This suggests that

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inhalation of adenosine A_{2A} receptor agonists would need to be the preferred route of administration, to achieve efficacy in the lung with an acceptable therapeutic index over systemic side effects.

The article will begin by discussing pre-clinical evidence supporting the anti-inflammatory role of the adenosine A_{2A} receptor in general and in the lung. Within this, mechanisms that mediate the anti-inflammatory effects *in vitro* and *in vivo* will be discussed. Subsequently this article will then discuss two recent clinical trials where an inhaled adenosine A_{2A} agonist had no obvious benefit in allergic rhinitis and asthma. The article will conclude with a discussion on the potential reasons for the lack of efficacy in these clinical trials and suggest that a more 'lung-focussed' adenosine A_{2A} receptor agonist is needed to fully explore the concept. The *in vitro* and *in vivo* biology of UK371,104 will be presented as an example of an adenosine A_{2A} agonist with a lung focus of pharmacological activity following intra-tracheal administration in a pre-clinical model of lung function.

The anti-inflammatory potential of adenosine A_{2A} receptor agonists

In vitro *evidence*

The adenosine A_{2A} receptor is expressed on virtually all cells that are implicated in the inflammatory process such as neutrophils, monocytes, eosinophils, epithelium, endothelium, lymphocytes and NK cells (see Gessi *et al*., 2000) suggesting that this receptor may play a role in the inflammatory process. Initial evidence promoting the role of the adenosine A_{2A} receptor came from agonists selective for activating this receptor in particular, CGS21680 the first reported agonist which selectively activates this receptor (Hutchinson *et al*., 1989) and more latterly, other selective A_{2A} receptor agonists such as ATL146e (Sullivan *et al*., 2001). See Figure 1 for structures of CGS21680 and ATL146e. *In vitro* experiments have provided a wealth of data that support the broad-spectrum anti-inflammatory potential of adenosine A_{2A} receptor agonists in cells implicated in both COPD and asthma. For instance, in neutrophils adenosine A_{2A} agonists have been demonstrated to inhibit several pro-inflammatory functions such as superoxide, elastase, leukotriene B4, TNFα and VLA4 induction (Sullivan *et al*., 2001, 2004; Pouliot, 2007). In addition adenosine A_{2A} agonists have been shown to inhibit the phosphorylation of ZAP70, a critical step in T cell activation (Sevigny *et al*., 2007) and suppresses perforin and FASL-mediated cytotoxicity in lymphokine-activated killer cells (Raskovalova *et al*., 2006). Inhibitory effects have also been noted on eosinophil secretion (Reeves and Butchers, 1999; Bevan *et al*., 2007), mast cell migration (Duffy *et al*., 2007) and on monocyte secretion (Link *et al*., 2000). In addition adenosine A_{2A} receptor activation has been shown to suppress the pro-inflammatory signalling mechanisms of cytokines, toll-like receptor agonists and LPS in several cell types (for example, Link *et al*., 2000; Sands *et al*., 2004, 2006). Further work has also noted that adenosine A_{2A} receptor agonists promote wound healing (Allen-Gipson *et al*., 2006; Cronstein, 2006).

The adenosine A_{2A} receptor is a Gs-linked receptor elevating the intracellular levels of cyclic AMP and by interacting with several cyclic AMP-binding proteins (such as protein kinase A, PKA and exchange proteins directly activated by cyclic AMP-EPAC, see Sands and Palmer, 2008) elicit the broad-spectrum anti-inflammatory effects noted above. In leukocytes, PDE4 is the major phosphodiesterase-controlling cyclic AMP levels in these cells (Houslay *et al*., 2005). In support of this many publications have documented that the anti-inflammatory effects (for example, inhibition of superoxide release) can be blocked by selective inhibitors of PKA such as H-89 (for example, Sullivan *et al*., 2001; Raskovalova *et al*., 2006; Sevigny *et al*., 2007). In recent years, however, the molecular mechanisms by which adenosine A_{2A} agonists mediate their effects have expanded considerably. For instance adenosine A_{2A} agonists have been shown to inhibit JAK (Janus Kinase)-STAT (signal transducer and activator of transcription) pathway activation by

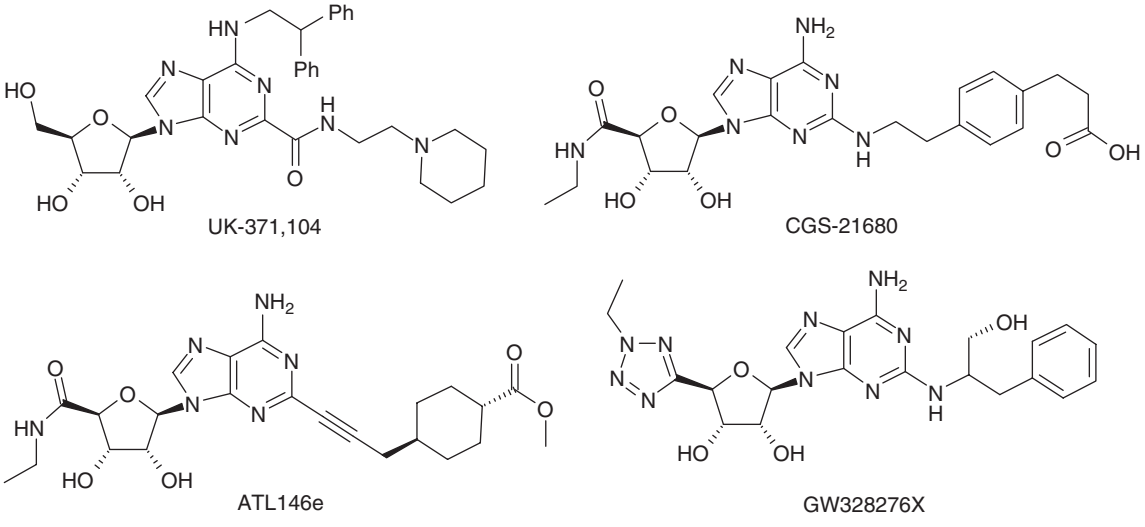

Figure 1 Structures of adenosine A_{2A} receptor agonists.

cytokines that utilize gp130 in their signalling mechanism through a PKA-independent mechanism because the effects are not blocked by H-89 but are inhibited with EPAC1 knockdown using siRNA (Sands *et al*., 2006). In this scheme, cyclic AMP activates EPAC 1 resulting in induction of SOC3 (suppressor of cytokine signalling) with subsequent inhibition of cytokine receptor signalling. Another major mechanism whereby adenosine A_{2A} receptor activation mediates an anti-inflammatory effect is to inhibit the nuclear factor-kappa B pathway (NF-κB) activated by pro-inflammatory agents such as cytokines and LPS independent of parallel pathways such as p38 (Minguet *et al*., 2005; Sands *et al*., 2006). The mechanism of A_{2A}-mediated effects on NF-κB differed according to the cell type studies. In glial cells the mechanism involved blockade of IKK-mediated IκBα phosphorylation that triggers degradation. In contrast, in HUVECs suppression of the NF-κB pathway it did not appear to involve IκBκ degradation (Sands *et al*., 2004). The adenosine A_{2A} receptor can also influence mitogen-activated protein kinases, which have been implicated in a wide variety of cellular responses involved in the inflammation process including apoptosis and several publications have documented ERK1/2 activation in a wide variety of cells. In CHO, cells induction of ERK expression by adenosine A_{2A} receptor activation is inhibited by H-89 but in HEK 293 cells induction appears to be independent of cyclic AMP (see Schulte and Fredholm, 2003). Inflammatory conditions are associated with hypoxia and this is associated with activation of hypoxia-inducible factor 1 (HIF1), inactivation of which leads to an impaired inflammatory response (Cramer *et al*., 2003). Adenosine A_{2A} receptor activation has been shown to increase HIF levels in macrophages suggesting another potential mechanism whereby adenosine A_{2A} receptors can control leukocyte function in hypoxic conditions (Lukashev *et al*., 2004a; de Ponti *et al*., 2007). In addition, pro-inflammatory mediators such as LPS, TNFα and IL1β have been shown to increase the expression of adenosine A_{2A} receptors in endothelium, macrophages and monocytes (Khoa *et al*., 2001; Nguyen *et al*., 2003; Murphree *et al*., 2005).

Two additional features of the adenosine A_{2A} receptor appear important for the broad-spectrum anti-inflammatory properties noted above. First, in contrast to other adenosine receptors, the adenosine A_{2A} receptor has a very long intracellular C-terminal and this has been suggested to be important in controlling the varied intracellular mechanisms of adenosine A_{2A} receptor activation. In addition, compared with other GPCRs, the adenosine A_{2A} receptor is quite resistant to agonist-induced desensitization and thus may aid the pro-inflammatory signalling *in vivo* (see Zezula and Freissmuth, 2008).

To conclude, the above *in vitro* evidence clearly identifies that the adenosine A_{2A} receptor has the potential of having an important and broad-spectrum of function in controlling inflammation. Key properties include the wide spectrum of intracellular signalling mechanisms that mediate the broad-spectrum anti-inflammatory actions demonstrated. In addition the upregulation of the receptor by pro-inflammatory mediators and the resistance to agonist-induced desensitization suggest a positive-feedback mechanism to maintain receptor function in the control of inflammation. For more information on this topic the reader is referred to the following reviews (Sullivan and Linden, 1998; Sitkovsky *et al*., 2004; Sands and Palmer, 2005; Fredholm *et al*., 2007; Palmer and Trevethick, 2008).

In vivo *evidence*

Three lines of *in vivo* experimental evidence targeting the A_{2A} receptor directly in animal models of inflammation–tissue damage support the *in vitro* observations by demonstrating: (1) application of selective agonists reduces inflammation and tissue damage, (2) administration of an A_{2A} receptor-selective antagonist enhances inflammation following induction of an inflammatory response and (3) inflammation is exaggerated in animals with targeted deletion of the adenosine A_{2A} receptor. Two groups have provided much of the evidence to be reviewed in this section and the reader is referred to publications from Joel Linden (University of Virginia) and Michail Sitkovsky (North Eastern University) and the following reviews (Sitkovsky, 2003; Lappas *et al*., 2005; Linden, 2005, 2006; Sitkovsky and Lukashev, 2005; Sitkovsky and Ohta, 2005).

Administration of adenosine A_{2A} receptor agonists have been shown to inhibit inflammation and tissue damage in a wide variety of *in vivo* models studying organs such as gut, heart, lung, liver, kidney, joints and the CNS (see Hasko and Pacher, 2008). Although supportive of the anti-inflammatory properties, use of receptor ligands *in vivo* has the potential to mislead as the receptor selectivity of these ligands for the corresponding animal adenosine receptors is not presented, making unequivocal conclusion of an A_{2A} receptor mechanism or the target cells for the anti-inflammatory action difficult. Some publications have confirmed the A_{2A} mechanism by demonstrating that either an adenosine A_{2A} antagonist or use of A_{2A}-deficient animals inhibits the protective action of selective adenosine A_{2A} agonists.

Exacerbation of tissue damage and inflammation with A_{2A} receptor antagonists has been shown in a variety of inflammatory models. For instance, treatment of mice with the selective A_{2A} receptor antagonist ZM241385 enhanced liver injury and inflammation in response to concanavalin A, *Pseudomonas aeruginosa* and carbon tetrachloride (Ohta and Sitkovsky, 2001; Chan *et al*., 2006). Similarly, ZM241385 prevented both the anti-inflammatory effects and the increased survival rates induced by low-dose ketamine administration, which promotes adenosine accumulation, in mice where sepsis was induced by LPS or *Escherichia coli* (Mazar *et al*., 2005). In the mouse lung, treatment with ZM241385 enhanced lung neutrophilia in response to intratracheal administration of LPS (Thiel *et al*., 2005). However, in other models, ZM241385 did not enhance inflammation/tissue damage in response to an inflammatory stimulus (see, for example, Peirce *et al*., 2001; Fozard *et al*., 2002). This may reflect either varying amounts of endogenous adenosine in the different models or, alternatively, administration of a near-maximal dose of inflammatory stimulus, which would make it difficult to observe any further enhancement with an A_{2A} receptor-selective antagonist.

In recent years, great impetus to the field of the anti-inflammatory effects of the adenosine A_{2A} receptor has come from the work of Sitkovsky and coworkers using A_{2A} receptor knockout mice. For instance, mice deficient in the A_{2A} receptor displayed an exaggerated inflammatory response and cytokine release in response to a wide variety of inflammatory insults, such as concanavalin A and carbon tetrachloride-induced liver damage and LPS-induced inflammation in an air pouch model (Ohta and Sitkovsky, 2001; Chan *et al*., 2006). In addition, adoptive transfer studies employing inflammatory cells from mice lacking the A_{2A} receptor have also supported the concept that this receptor functions as a physiological anti-inflammatory mechanism (Yang *et al*., 2006).

To conclude, pharmacological and molecular techniques support the concept that in an inflammatory environment the A_{2A} receptor functions as a 'physiological brake' on inflammatory processes and that agonism of this receptor by selective agonists might represent a novel anti-inflammatory mechanism. A number of reviews have been written, highlighting the concept that the A_{2A} receptor is involved in inflammation and tissue repair (for example, Sitkovsky, 2003; Hasko and Cronstein, 2004; Sitkovsky *et al*., 2004; Linden, 2005; Fredholm, 2007).

Anti-inflammatory role of the adenosine A_{2A} receptor in the lung: pre clinical studies

Despite the strong evidence supporting a role for A_{2A} agonists in controlling inflammation it is only comparatively recently that publications have appeared documenting the anti-inflammatory role of the adenosine A_{2A} receptor in the lung. Fozard *et al*. (2002) were the first to report that the adenosine A_{2A} receptor was an important regulator of lung inflammation. In this study, intra-tracheal administration of CGS21680 inhibited lung inflammation in brown Norway rats following sensitization and challenge with ovalbumen. This inhibition was measured as a reduction of eosinophils and neutrophils in the broncho–alveolar fluid (BALF) and this was reversed by the A_{2A} receptor antagonist ZM241385, supporting the A_{2A} mechanism of protection. In mouse models of lung inflammation (ovalbumen sensitization and challenge), Bonneau *et al*. (2006) reported that intranasal administration of CGS21680 inhibited infiltration of neutrophils, eosinophils, macrophages and lymphocytes into BALF. This suppression of inflammation was not associated with any change in hyper-reactivity of the airways as assessed by the action of nebulized methacholine on lung function (Penh) responses. In contrast, CGS21680 had no effect on leukocyte infiltration into the BALF in mice challenged with LPS or cigarette smoke. These latter models are primarily models of neutrophil infiltration and thus it is surprising given the effects on human leukocytes that no inhibition was observed. CGS21680 did, however, inhibit neutrophil activation as measured by inhibition of elastase release into the BALF. In the LPS model CGS21680 did appear to reduce the levels of pro-inflammatory cytokines (TNFα, KC and MIP-2) in the BALF although this did not reach statistical significance. Reasons for this weak effect in the LPS model are not clear but may reflect that higher doses of CGS21680 were needed to show efficacy in this model.

Further support has come from animals deficient in the adenosine A_{2A} receptor (A_{2A} knockouts). In wild-type mice, Reutershan *et al*. (2007) demonstrated that the selective A_{2A} receptor agonist ATL202 did inhibit LPS-induced neutrophil infiltration into the BALF. This contrasts with the findings of Bonneau *et al*. (2006) but may reflect methodology differences such as mice used (C57BL/6 versus BALBc) and route of administration of A_{2A} agonists (inhalation versus intranasal). In contrast, the protective effect of ATL202 was not evident in mice deficient in the A_{2A} receptor (Reutershan *et al*., 2007). Nadeem *et al*. (2007) demonstrated enhanced airway reactivity and lung inflammation in ragweed-sensitized mice lacking the adenosine A_{2A} receptor. This study demonstrated enhanced infiltration of lymphocytes, eosinophils and neutrophils in BALF of ragweed-challenged sensitized mice lacking the A_{2A} receptor together with hyper-reactive response to inhaled methacholine challenge as a measure by Penh lung function measurements.

Adenosine deaminase-deficient mice have enhanced levels of adenosine and in the lung this leads to a phenotype characterized by acute and chronic inflammation, mucin overproduction and angiogenesis (see Blackburn, 2003) due, in part, to activation of the low affinity adenosine A_{2B} receptor as evidenced by the protective effects of adenosine A_{2B} antagonists in this model (Sun *et al*., 2006). A counterpart to this should be that activation of the anti-inflammatory adenosine A_{2A} receptor should also lead to inhibition of lung inflammation in these mice. Adenosine deaminase-deficient mice which had genetic deletion of the adenosine A_{2A} receptor exhibited biology consistent with a key role for the adenosine A_{2A} receptor as a modulator of inflammation (Mohsenin *et al*., 2007). Adenosine deaminase/adenosine A_{2A} ($ADA^{-/-}$ $A_{2A}{}^{-/-}$) deficient animals died on average 3 days earlier than animals with normal levels of the A_{2A} receptor ($ADA^{-/-}$ $A_{2A}{}^{+/+}$) suggesting that this receptor plays a tissue protective role in this disease pathology in mice. Histology revealed that early death in $ADA^{-/-}$ $A_{2A}{}^{-/-}$ animals was associated with enhanced pulmonary inflammation (mainly macrophages) and mucin production, together with thickening of bronchial smooth muscle and hypertrophy of bronchial epithelium. The expression of chemokines CXCL1 and MCP1 were elevated in $ADA^{-/-}$ $A_{2A}{}^{-/-}$. These agents have been associated with neutrophil and macrophage recruitment respectively and in other *in vivo* systems are downregulated by A_{2A} receptor agonists (for example, Harada N *et al*., 2000). Not all pro-inflammatory molecules were elevated in $ADA^{-/-}$ $A_{2A}{}^{-/-}$ mice, for instance there was no elevation over $ADA^{-/-}$ $A_{2A}{}^{+/+}$ in TNFα and IL6 whereas transcripts for CCL11 and CCL17 were lower in $ADA^{-/-}$ $A_{2A}{}^{-/-}$. This might suggest that other adenosine receptors might be involved in this 'adenosine-driven' model of lung inflammation such as the A_{2B} receptor (Sun *et al*., 2006). An important observation in this study was the effects on angiogenesis. In $ADA^{-/-}$ $A_{2A}{}^{-/-}$ there was a significant increase in tracheal angiogenesis and in the chemokine CXCL1. The chemokine CXCL1 stimulates angiogenesis and has been suggested to play a role in chronic lung disease such as bronchiolitis obliterans (Belperio *et al*., 2002).

To conclude, three lines of evidence support the concept that the adenosine A_{2A} receptor can control inflammation in the lung. First is the inhibition of inflammation provided by treatment with selective agonists of the adenosine A_{2A} receptor, second are the observations that lung inflammation is enhanced in animals deficient in the A_{2A} receptor and third are observations that anti-inflammatory effects of the selective A_{2A} agonists CGS21680 and ATL202 are inhibited in A_{2A} receptor knockout animals or by pre-treatment with an A_{2A} receptor antagonist.

Anti-inflammatory role of the adenosine A_{2A} receptor in the lung: potential mechanisms of the anti-inflammatory effect

Four mechanisms have been proposed to explain the anti-inflammatory effects of adenosine A_{2A} agonists demonstrated from *in vivo* studies although, at present, few of these have been specifically studied in the lung. These are increases in oxygen supply/demand ratio, ischaemic preconditioning and post-conditioning, anti-inflammatory responses and angiogenesis (see Linden, 2005).

The anti-inflammatory mechanism can be discussed under two broad categories—first, are the biochemical mechanisms of the anti-inflammatory actions identified *in vitro* demonstrable in the lung? and second, what are the target cells/mediators, which might be pivotal for the anti-inflammatory actions for agonists of the adenosine A_{2A} receptor?

Few studies in the lung have investigated the signalling mechanisms underlying the anti-inflammatory effects of adenosine A_{2A} receptor agonists. Regulation of NF-κB activity involves nuclear translocation following release from the inhibitor IκBα, which is achieved by phosphorylation and degradation. Mice deficient in the adenosine A_{2A} receptor demonstrated increased phosphorylation of IκBα consistent with removal of the adenosine 'A_{2A} brake' on nuclear translocation of NF-κB (Nadeem *et al*., 2007). The increase in lung NF-κB was associated with increased gene expression in the lung of iNOS and NO production together with increase in lipid peroxidation. In support of this, Lukashev *et al*. (2004b) demonstrated that splenocytes from A_{2A}-deficient mice had increased levels of mRNA for a wide variety of cytokines following *in vivo* challenge with LPS and this was associated with an increase in NF-κB. In addition, in animals with normal levels of the adenosine A_{2A} receptor, the selective agonist CGS21680 was shown to inhibit an increase in LPS-induced mRNA cytokine expression. These observations in the lung thus support *in vitro* studies documenting the interaction of the adenosine A_{2A} receptor with the NF-κB pathway.

Mechanistically, however, the anti-inflammatory effect of the adenosine A_{2A} receptor in the lung is monitored through changes in leukocyte and cytokine levels (decreased through A_{2A} agonists and increased in A_{2A}-deficient animals). Two key questions thus arise—what target cells and cytokines are pivotal in the anti-inflammatory mechanism of the adenosine A_{2A} receptor? First though it is worth noting a clear gap in the literature on the anti-inflammatory effects of adenosine A_{2A} agonists on human versus animal leukocytes. Although many publications have clearly documented the anti-inflammatory properties of adenosine A_{2A} agonists on human isolated leukocytes, apart from equine neutrophils and monocytes (Sun *et al*., 2007, 2008), there are no publications documenting the anti-inflammatory effects of adenosine A_{2A} receptor agonists on isolated neutrophils from common laboratory animals. Indeed our own studies have failed to find any inhibitory effects of adenosine A_{2A} receptor agonists on mediator release from neutrophils isolated from rat, guinea pig, pig and dog stimulated with agents such as fMLP and LPS (results not shown). One publication has shown that CGS21680 produced a very small inhibition of H_2O_2 release from fMLP-stimulated granulocytes from lungs of mice with lung inflammation but not in neutrophils from non-inflamed environments (Thiel *et al*., 2005). There are several explanations for this anomaly: (a) SAR for activation of the leukocytes A_{2A} receptor is different to that of human, (b) studies have not measured the correct response in animal neutrophils to assess the effects of A_{2A} agonism or, (c) the receptor has to be induced to be expressed/activated. This latter hypothesis is supported by data from Thiel *et al*. (2005) although the data is far from conclusive. Thus in animal models of lung inflammation the neutrophil may not be a target cell underlying the anti-inflammatory action of adenosine A_{2A} receptor agonists and other cells must be involved such as monocyte/macrophages where there is evidence of anti-inflammatory effects of adenosine A_{2A} receptor agonists (for example, Kreckler *et al*., 2006).

Further understanding of the potential cell mediators involved in the anti-inflammatory action of the adenosine A_{2A} receptor agonists has come from studies assessing inflammatory response in other organs following ischaemia-reperfusion. Ischaemia-reperfusion injury (IRI) is associated with tissue damage during ischaemia and further injury following reperfusion. During reperfusion, tissue damage is associated with the generation of free radicals, cytokines, induction of adhesion molecules and infiltration of leukocytes into the tissue. Many studies have shown that the inhibitory action of adenosine A_{2A} agonists on IRI is because of an action of CD4+ T cells in orchestrating the inflammatory process such as neutrophil infiltration. For instance, infarct size in response to IR is smaller in Rag1 knockout mice, which lack mature lymphocytes, and is increased by adoptive transfer of CD4+ T cells from control animals and those lacking the A_{2A}AR but not in those T cells lacking the ability to secrete IFNγ. Importantly, agonism of the A_{2A} receptor, reduced both infarct size and inflammation in Rag1 knockout mice reconstituted with mature T cells but not in those reconstituted with CD4+ T cells lacking the A_{2A}AR (Yang *et al*., 2006). Likewise, in the kidney the protective effect of the selective adenosine A_{2A} agonist, ATL-146e was inhibited in mice whose bone marrow had been ablated and reconstituted with bone marrow from A_{2A} receptor-deficient animals. Using adoptive transfer techniques, the mechanism of protection of ATL-146e was shown to be because of an action of A_{2A} receptor agonists on $CD4^+$ T cells and involved an inhibition of IFNγ release (Day *et al*., 2003, 2006). Further mechanistic insights have come from studies of IRI in the liver. Lappas *et al*. (2006) noted that the

rapid induction of reperfusion injury was not consistent with the known time course for activation of conventional CD4+ T cell responses and suggested that injury must therefore be mediated by a rapidly activated T cell subset. Their studies demonstrated that CD1d-dependent NKT type 1 cells were key to initiating IRI. NKT type 1 cells resemble CD4+ Th2 cells and rapidly synthesize and release large quantities of cytokines but have an invariant T cell receptor that recognises glycolipids (see Meyer *et al*., 2008). Evidence to support this included (a) Depletion of NKT cells or administration of a CD1d-blocking antibody resulted in inhibition of IRI, (b) Transfer of NKT cells to NKT-depleted animals enhanced IRI and (c) neutrophil and IL13 elevations following IRI were significantly reduced in NKT-depleted animals. In this model A_{2A} receptor agonism inhibited IRI but this was prevented in NKT-deficient animals or in animals where NKT cells lacked the adenosine A_{2A} receptor. This was supported by *in vitro* observations documenting the inhibitory effect of A_{2A} receptor agonism on IL13 production form NKT cells. NKT cells account for a high percentage of liver T cells and thus an action of a tissue-localized cell (NKT type 1) appears to be pivotal for initiating the anti-inflammatory effects of A_{2A} agonists in this model.

CD1d is expressed in the epithelium of airways as well as inflammatory cells suggesting that this mechanism might be involved in the anti-inflammatory effects of adenosine A_{2A} receptor agonists in animal models of lung inflammation. Studies in animals suggest that these cells may be important for airway hyper-responsiveness (AHR) to methacholine as CD1d knockout animals or animals treated with CD1d-blocking antibody inhibited AHR and reduced (but not abolished) the inflammatory response (see Meyer *et al*., 2008 for a review). In asthma, lung inflammation is dominated by the presence of eosinophils and CD+ T cells and NKT cells have been found in the lung and some, but not all studies, show elevated levels of NKT cells in asthmatics compared with controls (see Ho, 2007; Meyer *et al*., 2008). Thus NKT cells may play an important role in lung inflammation.

Within the lung, activation of non-myelinated bronchopulmonary C fibres by various stimuli (such as capsaicin) in animal models can induce many of the symptoms of clinical asthma such as bronchoconstriction, apnea, hyperreactivity, cough and local inflammation (Coleridge and Coleridge, 1984). Likewise, inhalation of capsaicin induces bronchoconstriction and enhances hyper-reactivity in asthmatics. The mechanism is suggested to be through activation of the transient receptor potential (TRP) ion channel, which is expressed in human lung bronchpulmonary C fibres (see Jia and Lu-Yuan, 2007). In anaesthetized guinea pigs, Morimoto *et al*. (1993) demonstrated that capsaicin-induced increases in bronchoconstriction, substance P release and tracheal plasma extravasation could be inhibited by adenosine A2 agonists such as NECA and CGS21680. This suggests the involvement of the A_{2A} receptor in mediating this inhibitory response. Recent evidence has also shown that the A_{2A} agonist CGS21680 inhibits capsaicin-induced activation of TRPV1 stably expressed in HEK cells (Puntambekar *et al*., 2004). These data thus suggest that A_{2A} receptor agonism can suppress C fibre activation through inhibition of TRPV1.

There have been several publications documenting the wound-repairing properties of adenosine A_{2A} agonists in many organs including the lung (Allen-Gipson *et al*., 2006; Cronstein, 2006). Angiogenesis (growth of new blood vessels from pre existing vasculature) is an important mechanism in this process but little work has been published directly examining the lung. Nevertheless, angiogenesis is evident in the tracheas and lungs of patients with lung inflammation (Tanaka *et al*., 2003) and it has been suggested that this may contribute to airway hyper-reactivity/obstruction and may serve as new routes for inflammatory cell entry and tissue remodeling (Charan *et al*., 1997). The role of adenosine A_{2A} receptors in controlling lung angiogenesis is not fully elucidated but certainly angiogenesis is increased in adenosine deaminase-deficient mice, which lack the A_{2A} receptor (Mohsenin *et al*., 2007). In other tissues A_{2A} agonism stimulates angiogenesis and increases the rate at which wounds close through regulation of the angiogenic factor VEGF (for example, Montesinos *et al*., 2004) but other factors can also be involved such as downregulation of antiangiogenic factors such as thronmbospondin 1 in human umbilical vein endothelium (Desai *et al*., 2005). In addition, macrophages are converted from a pro-inflammatory phenotype to a pro-angiogenic phenotype that secretes VEGF (Olah and Caldwell, 2003) by the adenosine A_{2A} receptor and this may involve signalling through MyD88 to effect this switch (Macedo *et al*., 2007). Thus overall there is an emerging evidence about the role of adenosine and the A_{2A} receptor in promoting angiogenesis but more work is necessary to define the effects that this will have in the lung.

To conclude, by combining data from human isolated leukocytes with *in vivo* studies in animal models of inflammation, the mechanisms by which adenosine A_{2A} agonists inhibit the inflammatory process is beginning to clarify. Two major mechanisms appear to be involved. First is a direct inhibition of the pro-inflammatory properties of all the major leukocyte groups and recent data from animal models suggest that a tissue-localized cell (such as NKT cells) may be responsible for an early initiation step of the inflammatory process and subsequent recruitment of inflammatory cells into the tissue. Thus in an inflamed lung activation of the adenosine A_{2A} receptor would inhibit the pro-inflammatory effects of leukocytes and also inhibit a major initiation pathway (through NKT cells). In addition, the adenosine A_{2A} receptor has been shown to inhibit the activity of the TRPV1 receptor and subsequent effects on bronchoconstriction and inflammation. This, coupled with reports that adenosine A_{2A} agonist can promote wound healing, suggests that treatment with an adenosine A_{2A} agonist would inhibit inflammatory processes by at least three potential mechanisms: (a) inhibition of leukocyte pro-inflammatory properties, (b) through TRP inhibition, reduction in C fibre activity with potential effects on reducing bronchial hyper-reactivity and, (c) aid in the repair process. If these mechanisms do indeed occur in the human lung then this should be manifested as improvements in lung function (see Figure 4). Only two studies have measured lung function in animal models of inflammation. In A_{2A}-deficient animals (Nadeem *et al*., 2007) lung inflammation is exaggerated and this is coupled with a greater bronchoconstrictive

response to methacholine compared with normal animals. On the contrary, the inhibitory effects of CGS21680 on inflammation in rat lung were not accompanied by inhibition of hyper-reactivity to methacholine (Fozard *et al*., 2002). This lack of effect on lung function may reflect the action of CGS21680 at non-A_{2A} receptors (see Bonneau *et al*., 2006).

Anti-inflammatory role of the adenosine A_{2A} receptor: clinical studies in allergic rhinitis and asthma

Two clinical studies have recently been reported on the effects of the A_{2A} agonist GW328267X (Bevan *et al*., 2007) on allergic rhinitis (Rimmer *et al*., 2007) and on allergen-induced asthmatic response (Luijk *et al*., 2008). See Figure 1 for structure of GW328267X. These studies do not support the impressive pre-clinical studies documenting the anti-inflammatory profile outlined above. In allergic rhinitis, the effect of intranasal administration of GW328267X (50 μg two times daily) for 7 days was compared with fluticasone (200 μg two times daily). The A_{2A} agonist-induced a very small improvement in nasal blockage but had no effect on peak nasal inspiratory flow, rhinorrhoea or sneezes. In contrast, fluticasone significantly improved nasal blockage, peak nasal inspiratory flow and rhinorrhoea but had no effect on sneezes. GW328267X did produce a small but significant inhibition of tryptase and ECP whereas fluticasone inhibited ECP, IL5 and IL8. A similar lack of efficacy of GW328267X was also shown on the early and late asthmatic response in response to antigen (Luijk *et al*., 2008). In this study, inhalation of GW328267X (25 μg two times daily for 7 days) was compared with inhalation of fluticasone (250 μg bid). The A_{2A} agonist had no effect on the early or late asthmatic response or on any marker of inflammation measured. On the contrary, fluticasone totally inhibited both the early and late asthmatic response and some of the inflammatory markers measured such NO, eosinophils and ECP.

These studies cast a doubt on the concept that agonists of the adenosine A_{2A} receptor have clinical benefit despite the impressive benefits demonstrated in animal models of inflammation and lung function.

Is the adenosine A_{2A} receptor agonist concept flawed or is the idea still to be fully evaluated?

Assuming that adenosine A_{2A} agonists will provide clinical benefit, then two explanations could be advanced to explain the negative effects in the above studies. First is the lack of receptor selectivity of the agonist used and second, the lack of a sufficient therapeutic index. The receptor selectivity of agonists is often undertaken in human recombinant receptors and levels of receptor expression can radically alter the potential receptor selectivity identified. In consequence, therefore, actions at other adenosine receptors might have offset clinical benefits. For instance activation of the A_1 receptor has been shown to be pro-inflammatory in human isolated leukocytes, cause constriction of human isolated bronchi and enhance bronchoconstriction in anaesthetized guinea pigs through activity on sensory nerves (see Brown *et al*., 2008). In addition, there is some evidence to suggest that activation of the A_3 receptor can also cause bronchoconstriction and may also be anti-inflammatory but other work demonstrates opposite effects and much more work is needed to elucidate the function of A_3 receptors in inflammation (see Gessi *et al*., 2008). Finally there is also evidence to support the concept that antagonism of the A_{2B} receptor might also produce an anti-inflammatory effect (see Sun *et al*., 2006). In ligand-binding studies GW328267X was shown to bind with quite high affinity to A_{2A} and A_3 but was very much weaker at A_1 and A_{2B}. Follow up functional studies demonstrated A_{2A} agonism and A_3 antagonism. Given the known functional pharmacology of GW328267X it is likely therefore that the major pharmacological drive to exert an anti-inflammatory effect is A_{2A} receptor agonism.

It is known that the adenosine A_{2A} receptor is widely distributed and that activation can lead to a wide variety of responses such as hypotension (see Fredholm *et al*., 2001). Work by Fozard *et al*. (2002) was the first demonstration that an A_{2A} receptor agonist could inhibit lung inflammation when administered directly to the lung of the rat. This publication also documented that, at the doses inhibiting lung inflammation, there were falls in blood pressure and the authors suggested that a selective action of an A_{2A} agonist in the lung would be mandatory to avoid the systemic side effects associated with absorption of A_{2A} agonists into the periphery. In the clinical studies discussed above both stated that it might have been possible to achieve efficacy if higher amounts of compound could have been administered but this was not possible on account of the

Table 1 Comparison of inhibitory effects of UK371,104 and CGS21680 on release of pro-inflammatory mediators from human isolated neutrophils

Stimulus and assay	*UK371,104* IC_{50} nM	*CGS21680* IC_{50} nM
fMLP		
Superoxide	55 (40–75)	44 (30–65)
Elastase	126 (86–186)	67 (33–148)
Leukotriene B4	41 (16–107)	19 (13–34)
C5a		
Superoxide	30 (12–72)	25 (10, 62)
Elastase	28 (16–49)	23 (11–46)
LPS		
MIP1β	54 (20–146)	207 (108–396)
TNFα	150 (12–181)	115 (45–299)

Human neutrophils were obtained from healthy volunteers and isolated by density gradient centrifugation. All assays were carried out in 96-well format in HBSS buffer pH7.4. Compounds were pre-incubated with isolated neutrophils for 10 min, followed by stimulation with fMLP (30 nM–1 μM), C5a (10 nM) or LPS (10–100 ng ml^{-1}). Elastase and superoxide release were measured over 3 min post stimulation by chromogenic substrate cleavage and cytochrome *c* reduction, respectively. Leukotriene B_4 (LTB_4), TNFα and MIP-1β biosynthesis was measured post-stimulation by ELISA. IC_{50} is the concentration required to inhibit mediator release by 50%. UK-371,104 and CGS-21,680 produced near maximal inhibition of release of all mediators with similar potencies ($P>0.05$ unpaired *t*-test). Data are geometric mean with 95% CI with $n>6$ (fMLP) and >4 C5a and LPS studies.

narrow therapeutic index for GW328267X, which manifests as tachycardia and hypotension. Thus, in these two clinical studies, it is unclear whether a pharmacologically active dose of the compound was administered.

If, therefore, adenosine A_{2A} receptor agonists can indeed have clinical benefits in lung diseases such as asthma and COPD, strategies need to be developed to produce an adenosine A_{2A} agonist with a much superior therapeutic index.

UK371,104: an inhaled adenosine A_{2A} receptor agonist with a lung focus of pharmacological activity

Given the prophetic warning by Fozard *et al*. (2002) and the subsequent speculation on lack of efficacy of GW328267X in clinical trials, our strategy has been to design adenosine A_{2A} receptor agonists which have a focus of pharmacological activity in the lung in pre clinical models. Work with inhaled corticosteroids has suggested properties which can improve the therapeutic index for inhaled agents such as prolonged effect in the lung coupled with low oral bioavailability, rapid clearance and high plasma protein binding (Derendorf, 2007).

UK371,104 is a potent and selective agonist of the human adenosine A_{2A} receptor (Barnard *et al*., 2007; Mantell *et al*., 2008), which also inhibits mediator release from human isolated neutrophils (Table 1). In this regard, the overall *in vitro* anti-inflammatory profile of UK371,104 is very similar to CGS21680 (Table 1). See Figure 1 for structure of UK371,104.

A key property was to assess pharmacological activity in the lung and simultaneously assess the therapeutic index over the cardiovascular properties associated with activation of peripheral activation of adenosine A_{2A} receptors such as hypotension. We chose not to investigate these properties in animal models of lung inflammation for two reasons: (a) there is overwhelming evidence that adenosine A_{2A} agonists are anti-inflammatory in the lung and other organs and (b) standard *in vivo* assays of lung inflammation study the effects of an agent on inflammatory indices at a single time point and assessment of a therapeutic index (such as effects on

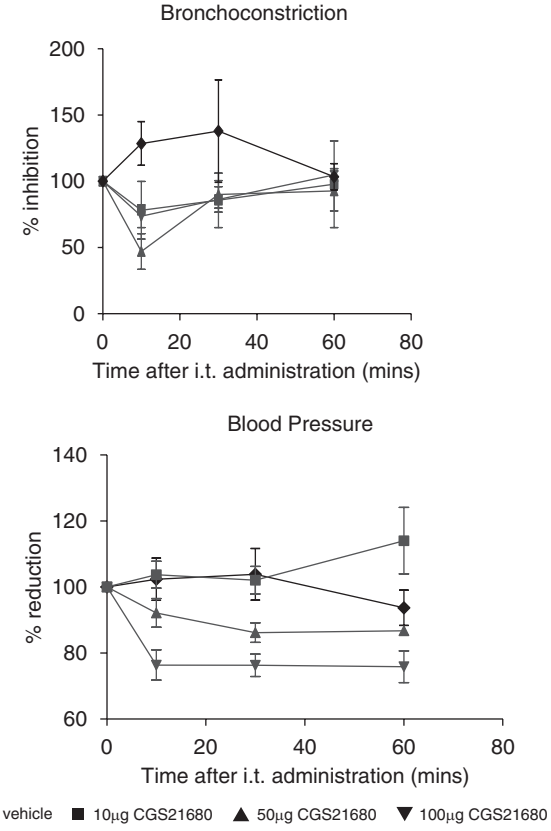

Figure 2 CGS 21680 inhibits capsaicin-induced bronchoconstriction and lowers blood pressure in the anaesthetized guinea pig. CGS21680 or solvent was administered in solution into the trachea (it) of anaesthetized guinea pigs. At the time points in the graphs above capsaicin was administered i.v. and the effects on lung resistance measured. Blood pressure was recorded just before administration of capsaicin. Values are mean with s.e.m. of at least four individual experiments. CGS21680 produced a small but significant inhibition of lung resistance at 15 min only at 50 and 100 μg intra-tracheal dose. Both of these doses caused a fall in blood pressure, which was significant at all time points with 100 μg and at 30 min only for 50 μg ($P<0.05$ unpaired *t*-test).

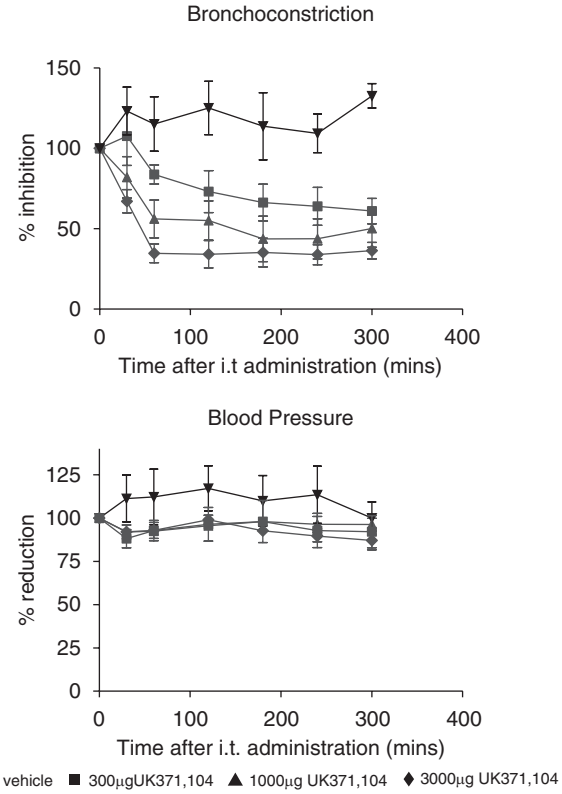

Figure 3 UK371,104 inhibits capsaicin-induced bronchoconstriction and has no effect on blood pressure in the anaesthetized guinea pig. UK371,104 or solvent was administered in solution into the trachea (it) of anaesthetized guinea pigs. At the time points in the graphs above capsaicin was administered i.v. and the effects on lung resistance measured. Blood pressure was recorded just before administration of capsaicin. Values are mean with s.e.m of at least four individual experiments. UK371,104 produced significant inhibition of lung resistance at all time 1–5 h post-administration ($P<0.05$ unpaired *t*-test) there was no effect on blood pressure at any dose ($P>0.05$ unpaired *t*-test).

blood pressure) is difficult in these models. Thus to simultaneously assess the effect of an A_{2A} agonist in the lung and cardiovascular system we chose to use a model originally described by Morimoto *et al*. (1993) in the anaesthetized guinea pig. In this model, adenosine analogues were demonstrated to inhibit capsaicin-induced bronchoconstriction by inhibiting substance P release. The mechanism is through inhibition of TRPV1 activity and inhibition of this receptor has been suggested as a route to control hyper-reactivity and some inflammatory responses within the lung (see Jia and Lu-Yuan, 2007).

Thus we investigated the potency, efficacy and duration of action of intra-tracheal administration of UK371,104 and CGS21680 on changes in heart rate, blood pressure and on capsaicin-induced bronchoconstriction in the anaesthetized guinea pig. Results with CGS21680 agreed with the observations of Fozard *et al*. (2002) in that CGS21680 inhibition of the capsaicin response in the lung was associated with falls in blood pressure. In contrast, UK371,104 inhibited the effects of capsaicin on lung resistance but had no effect on blood pressure (see Figures 2 and 3). In addition the duration of action of UK371,104 in the lung was considerably longer than that of CGS21680. This suggests that UK371,104 has a focus of pharmacological activity (and a long duration of action) in the lung following intra-tracheal administration (Figure 4).

The cardiovascular side effects following intra-tracheal administration of adenosine A_{2A} agonists are likely to reflect the maximum free plasma concentration (C_{max}). Thus this suggests that the free C_{max} of UK371,104 was much lower than CGS21680. This was assessed in parallel studies in the rat (conscious male rats, 250 g) by comparing the normalized C_{max} (free C_{max}/dose) of both compounds. At a normalized intra-tracheal dose of 1 mg/kg, the free systemic C_{max} of CGS21680 was 271 nM whereas that for UK371,104 was 20 nM. This concentration of CGS21680 is much higher than that required to activate the human A_{2A} receptor, in contrast, levels of UK371,104 are lower than those required to activate this receptor. Properties of UK 371,104 aiding this desirable low free C_{max} compared with CGS21680 are increased lipophilicity, molecular weight, clearance *in vivo* and increased plasma protein binding (Mantell *et al.*, 2008). Preliminary studies in our laboratory have also shown that at a normalized intra-tracheal dose of 1 mg kg^{-1} the free systemic C_{max} of GW328267X was significantly in excess of that required to activate the human A_{2A} receptor (results not shown).

Summary and perspectives on treating lung inflammation with inhaled agonists of the adenosine A_{2A} receptor

The adenosine A_{2A} receptor has an impressive pedigree as a potential anti-inflammatory agent. Adenosine A_{2A} agonists exhibit broad-spectrum anti-inflammatory effect *in vitro* on

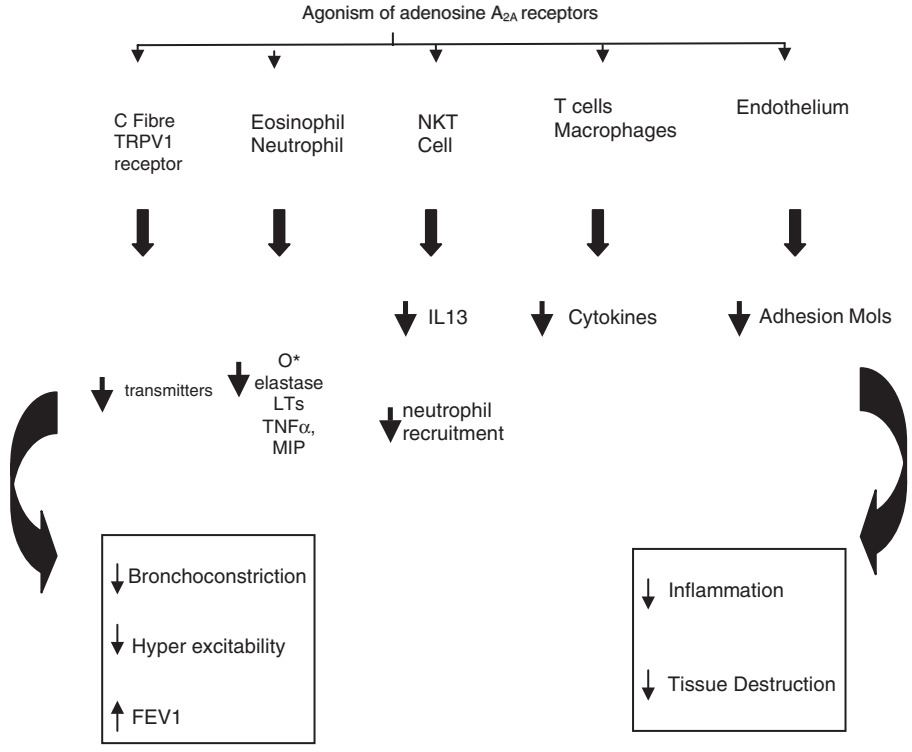

Figure 4 Potential mechanisms whereby adenosine A_{2A} receptor agonism can inhibit inflammatory processes in the lung. At least three mechanisms can be proposed: (1) inhibition of leukocyte proinflammatory properties of NKT cells, other leukocytes and endothelium, (2) reduction in C fibre activity by TRPV1 inhibition and (3) aid in the repair process. This should manifest as decreases in AHR (airway hyper reactivity also known as airway hyper excitability), improvements in lung function accompanied by less tissue destruction and potential to aid in the repair process. FEV1, forced expiratory volume in 1 second; O*, superoxide; TNFα, tumour necrosis factor α; MIP, macrophage inflammatory protein; NKT, natural killer T cell; LTs, leukotrienes; TRPV1, transient receptor potential vanilloid 1.

virtually all human cells involved in the inflammatory process. This is strongly supported by the anti-inflammatory actions of adenosine A_{2A} receptor agonists in a wide variety of *in vivo* models including the lung. The very wide spread distribution of the adenosine A_{2A} receptor suggests that, as far as the lung is concerned, inhalation would be the preferred route of administration to reduce the systemic side effects associated with activating this receptor. The lack of efficacy of inhaled GX328267X in clinical trials may be on account of the fact that the inhaled dose was too low and that the noted side effect profile (hypotension, tachycardia) precluded investigating higher inhaled doses. Using strategies similar to that devised to improve the therapeutic index of inhaled corticosteroids, the adenosine A_{2A} agonist, UK371,104 demonstrated efficacy in the lung without any obvious effects on blood pressure. Such 'lung-focussed' compounds may be valuable to study the anti-inflammatory potential of inhaled adenosine A_{2A} agonists in clinical trials. UK432,097 is an analogue of UK371,104 with a similar lung focus of pharmacological activity in the anaesthetized guinea pig model discussed above. UK432,097 is currently in phase II trials for COPD.

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Conflict of interest

All authors are employees of Pfizer Inc. Pfizer do not sell any of the drugs mentioned in the article (Figure 1). UK432,097 is currently in phase II trials for COPD.

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