Effect of a Specific and Selective A2B Adenosine Receptor Antagonist on Adenosine Agonist AMP and Allergen-Induced Airway Responsiveness and Cellular Influx in a Mouse Model of Asthma

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ABSTRACT

It has been previously proposed that adenosine plays an important role in the pathogenesis of asthma. The proposed mechanism of action for nucleoside adenosine is to activate A2B adenosine receptors (AR) and to indirectly modulate levels of mediators in the lung. In vivo data supporting the role of A2B AR in airway reactivity and inflammation in allergic animal models are lacking. The present study describes the effects of a selective A2B AR antagonist, CVT-6883 [3-ethyl-1-propyl-8-[1-(3-trifluoromethylbenzyl)-1H-pyrazol-4-yl]-3,7-dihydropurine-2,6-dione], on airway reactivity and inflammation in an allergic mouse model of asthma. Mice were sensitized with ragweed (i.p.) on days 1 and 6 and challenged with 0.5% ragweed on days 11, 12, and 13. On day 14, enhanced bronchial hyper-reactivity was observed in sensitized mice, and the effect was significantly attenuated by either CVT-6883 (1 mg/kg i.p.) or montelukast (1 mg/kg i.p.). Allergen challenge induced late allergic response in sensitized mice, which was inhibited by CVT-6883 (1 mg/kg i.p.). Allergen challenge also increased the number of cells in bronchoalveolar lavage fluid obtained from sensitized mice, and that was reduced by either CVT-6883 (6 mg/ml aerosolization for 5 min) or theophylline (36 mg/ml aerosolization for 5 min). These results suggest that A2BAR antagonism plays an important role in inhibition of airway reactivity and inflammation in this model of allergic asthma.

Evidence for the potential role of adenosine in the pathogenesis of asthma has been growing steadily since the early observation of its bronchoconstrictor levels in human asthmatics. In the early 1980s, it was shown that adenosine or AMP (which is converted to adenosine in vivo by 5'-nucleotidase) induces bronchoconstriction in asthmatics but not in normal subjects (Cushley et al., 1983). In contrast to methacholine, which induces changes in airway caliber, AMP-induced bronchial hyper-responsiveness is proposed to be related to the inflammatory status of the asthmatic lung (Spicuzza et al., 2006). Adenosine has also shown to increase the concentrations of mediators released from mast cells, such as histamine, tryptase, leukotriene C4, and prostaglandin G2 (Crimi et al., 1997). Adenosine-induced bronchoconstriction is attenuated by drugs that either inhibit mast cell activation or serve as antagonists to the mediators released from the mast cells (Holgate, 2005). Thus, a potential mechanism by which adenosine causes bronchoconstriction is mast cell activation (Polosa, 2002; Holgate, 2005). In addition, it has been shown that the concentration of adenosine in the bronchoalveolar lavage fluid (BALF) of patients with asthma is significantly higher than in normal subjects (Cushley et al., 1983). Therefore, adenosine is thought to play a role in the pathogenesis of asthma.
with asthma is higher than that in nonasthmatics (Driver et al., 1993). Higher concentrations of adenosine were detected in the exhaled breath condensate of atopic asthmatics compared with those of nonatopic controls (Huszar et al., 2002). Hence, adenosine may function as a paracrine mediator of the inflammatory responses in the lung.

The effects of adenosine are mediated through a family of cell surface G-protein-coupled receptors, which are currently classified into four adenosine receptor subtypes: A₁, A₂A, A₂B, and A₃. The roles of A₁ and A₂A ARs in the cardiovascular system have been well established (Shryock and Belardinelli, 1997), whereas the role of A₃ AR is less well understood. For the A₂B AR, recent studies have suggested that the A₂B AR may play an important role in mediating airway reactivity and modulating chronic inflammatory responses in the lung. For example, adenosine via activation of A₂B AR increases the release of inflammatory cytokines, such as IL-4, IL-8, and IL-13 from human mast cells (Feoktistov and Biaggioni, 1995; Feoktistov et al., 2001; Ryzhov et al., 2004), and these cytokines can induce IgE synthesis by B lymphocytes (Ryzhov et al., 2004). Likewise, adenosine activation of A₂A AR increases the release of inflammatory cytokines from human bronchial smooth muscle cells, human lung fibroblasts, and human airway epithelial cells (Zhong et al., 2004, 2005). These cytokines, in turn, induce differentiation of lung fibroblasts into myofibroblasts (Zhong et al., 2005) and increase the release of tumor necrosis factor α from monocytes (Zhong et al., 2006). These effects of adenosine have been shown to be inhibited by selective antagonists of the A₂B AR (Feoktistov and Biaggioni, 1995; Feoktistov et al., 2001; Ryzhov et al., 2004; Zhong et al., 2004, 2005, 2006). Thus, A₂B ARs may play an important role in the pathophysiology of asthma.

The allergic mouse model developed and characterized in this laboratory has been used to further understand the role of adenosine and its receptors in airway reactivity and inflammation (Fan and Mustafa, 2002, 2006). The previously described features of this model are indicated. 1) Aerosolized adenosine causes concentration-dependent bronchoconstriction, measured as Penh in sensitized mice, and 2) aerosolized adenosine potentiates the allergen-induced airway inflammation and both of these effects are blocked by theophylline at therapeutic concentrations (Fan and Mustafa, 2002, 2006). CVT-6883 is a specific and selective antagonist to the A₂B AR. Its binding affinities for the four subtypes of ARs were determined using competition radioligand binding assays in membranes isolated from cell lines that overexpress each of the four ARs (Sun et al., 2006). The aims of this study were to determine the effect of CVT-6883 on the airway reactivity induced by aerosolized NECA (5′-N-ethylcarboxamidoadenosine), AMP, or allergen and on the numbers of inflammatory cells in BALF after allergen challenge.

Materials and Methods

Animals

Male BALB/c mice, 6 to 8 weeks of age and free of specific pathogens, were obtained from Harlan Laboratories (Indianapolis, IN). The animals were maintained on a ragweed-free diet. All experimental animals used in this study were under an approved protocol from the Institutional Animal Care and Use Committee of East Carolina University.

Chemicals and Reagents

Ragweed pollen extract was purchased from Greer Laboratories (Lenoir, NC). Imject Alum was purchased from Pierce Laboratories (Rockford, IL). Theophylline was purchased from Sigma Chemical Co. (St. Louis, MO), and montelukast sodium was a gift from Merck and Co., Inc. (West Point, PA). NECA was purchased from Sigma Chemical Co. Diff-Quik stain set was purchased from Dale Behring Inc. (Newark, DE).

CVT-6883 was synthesized and provided by CV Therapeutics, Inc. (Palo Alto, CA). For i.p. injection, CVT-6883 was dissolved in DMSO and diluted in saline; thus, the i.p. vehicle of CVT-6883 is DMSO/saline (25/75, v/v).

Experimental Protocols

Sensitization and Challenge with Ragweed. Sensitization and challenge with ragweed (SENS) or saline (control) were performed according to a method described previously (Fan and Mustafa, 2002, 2006).

Measurement of Airway Reactivity. On day 14, mice were challenged with NECA, AMP, or allergen, and the airway reactivity was measured using barometric plethysmography (Fan and Mustafa, 2002, 2006; Oldenburg and Mustafa, 2005). It has been previously shown that changes (increases or decreases) in Penh correlate with changes (increases or decreases) in airway resistance in this model (Justice et al., 2001).

NECA Challenge. SENS mice were used in this study. There were four treatment groups: vehicle (DMSO/saline, 25/75, v/v, i.p.) and three concentrations of CVT-6883-treated groups (0.4, 1.0, and 2.5 mg/kg i.p.). On day 14, CVT-6883 or vehicle was given by i.p. injection 15 min before NECA challenge. NECA was dissolved in ethanol and diluted in saline; thus, the final NECA solution contained ethanol/saline (10/90, v/v). For NECA challenge, mice were placed in the Plexiglas chambers and exposed to the nebulization vehicle (ethanol/saline, 10/90, v/v) or increasing concentrations of NECA (46.9, 93.8, 187.5 and 375.0 μg/ml) for 2 min with an aerosol delivery system (version 1.5; Buxco, Sharon, CT) at 2.5 l/min of the dilution flow and 0.15 l/min of the trickle flow. Recordings of pressure fluctuations in the chamber were taken for 5 min after each nebulization. The next concentration of NECA was not given until the Penh values returned to baseline values. Airway reactivity was expressed as percentage increase in Penh compared with Penh values from the nebulization vehicle (ethanol/saline, 10/90, v/v) or increasing concentrations of NECA (46.9, 93.8, 187.5 and 375.0 μg/ml) for 2 min with an aerosol delivery system (version 1.5; Buxco, Sharon, CT) at 2.5 l/min of the dilution flow and 0.15 l/min of the trickle flow. Recordings of pressure fluctuations in the chamber were taken for 5 min after each nebulization. The next concentration of NECA was not given until the Penh values returned to baseline values. Airway reactivity was expressed as percentage increase in Penh compared with Penh values from the nebulization vehicle (ethanol/saline, 10/90, v/v).

AMP Challenge. Both control mice and SENS mice were used in this study. There were six groups of animals: control mice treated with the vehicle (DMSO/saline, 25/75, v/v, i.p.), CVT-6883 (1 mg/kg i.p.), or montelukast (1 mg/kg i.p.) and sensitized mice treated with the vehicle (DMSO/saline, 25/75, v/v, i.p.), CVT-6883 (1 mg/kg i.p.), or montelukast (1 mg/kg i.p.). On day 14, the vehicle, CVT-6883, or montelukast was given 15 min before AMP challenge. AMP was dissolved in saline. For AMP challenge, mice were placed in the Plexiglas chambers and exposed to the nebulization vehicle (saline) or increasing concentrations of AMP (6, 12, and 24 mg/ml in saline) for 2 min. The remaining procedure was the same as described above for NECA challenge. Airway reactivity was expressed as percentage increase in Penh compared with the nebulization vehicle (saline).

Allergen Challenge: Airway Reactivity. Both control mice and SENS mice were used in this study. There were three groups: control mice treated with the vehicle (DMSO/saline, 25/75, v/v, i.p.), sensitized mice treated with the vehicle, or sensitized mice treated CVT-6883 (1 mg/kg i.p.). On day 14, the vehicle or CVT-6883 was given 60 min before allergen challenge. For allergen challenge, mice were placed in Plexiglas chambers and exposed to either 5% ragweed or saline for 10 min with a nebulizer (DevIlbiss, Somerset, PA) at 2.0 ml/min, and the aerosol particles had a median aerodynamic diameter of less than 4 μm. Penh was recorded for 5 h with every 5-min interval. Late allergic response was calculated using the area under the curve (AUC_3–4 h).
Allergen Challenge: Inflammatory Cells in the BALF. Both control mice and SENS mice were used in this study. There were four groups: control or sensitized mice treated with the vehicle, CVT-6883 (6 mg/ml, aerosol for 5 min), or theophylline (36 mg/ml, aerosol for 5 min). The dose of theophylline was chosen based on our previous work (Fan and Mustafa, 2002), and the dose of CVT-6883 (6 mg/ml) was the highest soluble concentration in the vehicle. On day 14, 15 min after administration of CVT-6883 or theophylline, mice were exposed to either 2% ragweed or saline for 10 min with a DeVilbiss nebulizer at 2.0 ml/min, and the aerosol particles had a median aerodynamic diameter of less than 4 μm.

Five hours after the allergen challenge, BALF were collected and analyzed to assess airway inflammation. Mice were killed using i.p. injection of 0.1 ml of pentobarbitone sodium (200 mg/ml). The trachea was cannulated to introduce 0.8 ml of phosphate-buffered saline into the lungs three times, followed by centrifugation at 1500 rpm for 6 min at 4°C (model TJ-6 centrifuge; Beckman Instruments, Palo Alto, CA). The number of cells recovered per mouse was calculated and expressed as the mean ± S.E.M/milliliter for each group.

Statistical Analysis. Data were expressed as mean ± S.E.M. Data were analyzed by analysis of variance (ANOVA) followed by Tukey’s test for multiple comparisons. Paired t test was used for calculating difference in Penh values before and after and different drug treatments. A p value of <0.05 was considered statistically significant.

Results

Effect of CVT-6883 on NECA-Induced Increases in Penh. The effect of CVT-6883 on NECA-induced airway reactivity in SENS mice was determined. As shown in Fig. 1, aerosolized NECA increased Penh values in a concentration-dependent manner. The increases in Penh (percentage of nebulization vehicle) induced by NECA (46.9, 93.8, 187.5, and 375.0 μg/ml) were 86.00 ± 9.07, 136.38 ± 18.42, 200.48 ± 13.37, and 340.66 ± 46.64%, respectively (n = 6). Treatment with CVT-6883 (0.4, 1.0, or 2.5 mg/kg i.p., 15 min before NECA challenge) with either CVT-6883 (1 mg/kg i.p.) or montelukast (1 mg/kg i.p.) attenuated the AMP-induced increases in Penh. The AMP-induced increases in Penh in the SENS+Vehicle group was significantly greater than increases in Penh in SENS+CVT-6883 group (12 and 24 mg/ml AMP) or SENS+montelukast group (6, 12, and 24 mg/ml AMP) (p < 0.05, ANOVA).

Effect of CVT-6883 on Late Allergic Response. The effect of CVT-6883 on the airway reactivity induced by aller-

![Fig. 1. Effect of CVT-6883 on NECA-induced increases in Penh in sensitized mice. Mice were treated with vehicle (SENS + Vehicle) or three doses of CVT-6883 (SENS + CVT-6883). Data shown are mean ± S.E.M. (n = 5–6). The Penh values in the vehicle-treated group were significantly different from CVT-6883-treated groups (p < 0.05, ANOVA), except that there was no statistical difference between vehicle- and CVT-6883-treated (2.5 mg/kg) groups at NECA concentration of 93.8 μg/ml.](Image 1)

![Fig. 2. Effect of CVT-6883 and montelukast on AMP-induced increases in Penh in control and SENS mice. Data represent the concentration-response relationship to aerosolized AMP in control (a), and sensitized (SENS) mice (b) treated with vehicle, CVT-6883 (1 mg/kg i.p.), or montelukast (1 mg/kg i.p.). Data shown are mean ± S.E.M. (n = 7–8). The AMP-induced Penh increases in the SENS+vehicle group were significantly higher than Penh increases in SEN+CVT-6883 group (12 and 24 mg/ml AMP) or SEN+montelukast group (6, 12, and 24 mg/ml AMP) (p < 0.05, ANOVA).](Image 2)
gen in the SENS mice was also determined. As shown in Fig. 3a, late allergic response to allergen challenge was observed in sensitized but not in control mice. Treatment (60 min before allergen challenge) with CVT-6883 (1 mg/kg i.p.) attenuated this late allergic response (Fig. 3a, top).

To quantify this late allergic response, the area under the AUC Penh was calculated for the whole 5-h period, as well as for late allergic response that occurred between 3 and 4 h after allergen challenge. The percentage increases in AUC_{3–4 h} for SENS+Vehicle and SENS+CVT-6883 groups were approximately 106 and 7%, respectively, compared with control (n = 8; Fig. 3c). The AUC_{0–5 h} (Fig. 3b) and AUC_{3–4 h} (Fig. 3c) values of the SENS+Vehicle group were significantly higher than those of the control group, which was attenuated by treatment with CVT-6883 in sensitized group (SENS+CVT-6883), only in AUC_{3–4 h} (p < 0.05, ANOVA) as shown in Fig. 3c.

Effect of Aerosolized CVT-6883 and Theophylline on Allergen-Induced Increases in the Number of Inflammatory Cells in BALF. The effect of CVT-6883 on allergen-induced increases in the number of inflammatory cells in BALF obtained from sensitized mice was determined. As shown in Fig. 4 (a, top), allergen challenge significantly increased the number of total cells in BALF of sensitized group (SENS+Vehicle) compared with control group and treatment with either CVT-6883 (aerosol delivery of 6 mg/ml solution for 5 min), or theophylline (THEO, aerosol delivery of 36 mg/ml solution for 5 min) significantly reduced the increases in total cells (n = 6–8; Fig. 4a; p < 0.05, ANOVA).

Likewise, allergen challenge increased the number of eosinophils in BALF of the sensitized group (SENS+Vehicle) compared with control group, and treatment with either CVT-6883 or theophylline significantly reduced the increases in eosinophils (n = 6–8; Fig. 4b; p < 0.05, ANOVA).

In addition, allergen challenge also increased the number of lymphocytes in BALF of sensitized group (SENS+Vehicle) compared with control group; only CVT-6883 significantly attenuated the allergen-induced increase in lymphocytes (n = 6–8; Fig. 4c; p < 0.05, ANOVA).

Furthermore, allergen challenge increased the number of...
macrophages in BALF of sensitized group (SENS+Vehicle) compared with control group ($p < 0.05$, ANOVA); treatments either with theophylline or CVT-6883 did not significantly reduce the increased number of macrophage by allergen ($p > 0.05$, ANOVA). The numbers of macrophages ($10^5$) in control, SENS, SENS+THEO, and SENS+CVT-6883 groups were 10.05 ± 0.89, 19 ± 3.21, 24 ± 2, and 19 ± 2, respectively ($n = 6–8$).

**Discussion**

The main objective of this study was to determine the effects of $A_{2A}$ AR antagonist CVT-6883 on airway reactivity induced by AMP, NECA, or allergen, as well as allergen-induced infiltration of inflammatory cells in the mice airway. The major findings of this study were that treatment with CVT-6883 attenuated the airway reactivity induced by NECA, AMP, or allergen in sensitized mice. In addition, treatment with aerosolized CVT-6883 attenuated the increases in the number of total cells present in BALF, specifically eosinophils and lymphocytes after allergen challenge. Compared with montelukast, CVT-6883 was as effective as montelukast in inhibiting AMP-induced airway reactivity in sensitized mice. Moreover, CVT-6883 showed overall better efficacy in inhibiting allergen-induced influx of inflammatory cells into the lung compared with theophylline.

The acute effect of inhaled adenosine (or inhaled AMP) on bronchoconstriction is well established in asthmatic subjects (Holgate, 2005). One of the proposed mechanisms of AMP challenge suggests that adenosine, degraded from AMP, specifically eosinophils and lymphocytes after allergen challenge. Compared with montelukast, CVT-6883 was as effective as montelukast in inhibiting AMP-induced airway reactivity in sensitized mice. Moreover, CVT-6883 showed overall better efficacy in inhibiting allergen-induced influx of inflammatory cells into the lung compared with theophylline.

The acute effect of inhaled adenosine (or inhaled AMP) on bronchoconstriction is well established in asthmatic subjects (Holgate, 2005). One of the proposed mechanisms of AMP challenge suggests that adenosine, degraded from AMP, interacts with $A_{2B}$ receptors on the “primed” mast cells in the lung with subsequent release of preformed and newly formed mediators. The mediators in turn act on bronchial smooth muscle to cause bronchoconstriction (Holgate, 2005). Although this proposed mechanism seems to explain most of the clinical observations caused by inhalation of AMP, this mechanism remains unproven.

To determine which adenosine receptor subtype(s) are involved in adenosine-induced airway reactivity, several selective adenosine agonists or antagonists have been tested in numerous allergic animal models. Using the allergic mouse model in the current study, the selective $A_1$ agonist CPA (N6-cyclopentyladenosine) or CVT-510 [2-6-[(2S,3S,4R)-5-(hydroxymethyl)oxolane-3,4-diol] or selective $A_{2A}$ agonists CGS-21680 [2-[(2-carboxyethyl)-phenethylamino]-5’-N-ethylcarboxamidoadenosine] or CVT-3146 [1-[(4S,3R,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolane-2-yl]-6-amino-2-yl]pyrazol-4-yl]-N-methylcarboxamide] do not influence airway reactivity due to allergen challenge (data not shown).

In contrast, the nonselective agonist NECA increases airway reactivity, and the $A_3$ agonist CI-IB-MECA [N6-(3-iodo-benzyl)-2-chloro-adenosine-5’-N-methyluronamide] also increases airway reactivity albeit to a less extent than NECA (Fan et al., 2003; Fan, S. J. Mustafa, D. Zeng, and L. Belardinelli, unpublished data). The effect of NECA is partially blocked by enprofylline, a relative selective $A_{2B}$ antagonist, or MRS 1523, a selective $A_3$ antagonist. In allergic guinea pig model, it was reported that $A_2$ agonist CPA induces airway obstruction by a neuronal-dependent mechanism, whereas $A_{2A}$ agonist CGS-21680 or $A_3$ agonist IB-MECA has no effect (Keir et al., 2006). Interestingly, it was also reported that $A_3$ agonist IB-MECA could contract sensitized guinea pig trachea (Martin and Broadley, 2002). In allergic rabbit model, adenosine-induced contractions of tracheal and bronchial smooth muscles are mainly due to the activation of $A_1$ receptors on rabbit smooth muscle (Ali et al., 1994). In this model, $A_1$ antagonist L-97-1 seems to be effective in blocking adenosine-induced bronchoconstriction (Obiefuna et al., 2005). Obviously, different receptor subtypes have been implicated in airway reactivity depending on the animal models.

In the present study, similar to human, AMP challenge caused increase in airway reactivity in mice that were sensitized and challenged by ragweed but not in sham-sensitized/challenged mice. In addition, the AMP-induced airway reactivity was completed inhibited by treatment of CVT-6883. To our knowledge, this is the first report that a selective $A_{2B}$ antagonist is able to attenuate the AMP-induced airway reactivity in an animal model. The result supports the proposed mechanism that $A_{2B}$ receptors might be involved in mediating the airway response induced by AMP challenge.

However, due to the differences in airway physiology between human and animal models, one needs to be cautious in extrapolation of these findings to humans.

Because AMP-induced bronchoconstriction has been hypothesized to be an indirect mechanism by releasing contractile mediators, many clinical studies have focused on identifying potential mediators (Holgate, 2005). It has been shown that AMP-induced acute bronchconstriction can be inhibited by selective histamine H1 antagonist terfenadine or astemizole, leukotriene receptor antagonist montelukast, and inhibitors of cyclooxygenases 1 and 2 (indomethacin or flurbiprofen). In addition, AMP challenge leads to increased levels of several contractile mediators, including histamine, prostaglandin D2 in asthmatic airways, and leukotriene in breast condensate (Polo et al., 1995; Bucchiioni et al., 2004). In the present studies, CVT-6883 and montelukast attenuated AMP-induced airway reactivity to a similar degree, supporting the hypothesis that AMP-induced airway reactivity is mediated mainly via cysteinyl leukotrienes in this animal model.

In asthmatics, besides the acute bronchoconstriction, allergen induces a prolonged late-phase reaction due to the accumulation of cytokines and chemokines generated by resident inflammatory cells (e.g., mast cells, macrophage, and epithelial cells) and recruited inflammatory cells (e.g., lymphocytes and eosinophils) (Busse and Lemanske, 2001). In the present study, CVT-6883 inhibited the late-phase allergen-induced airway reactivity and inhibited the allergen-induced increase in eosinophils and lymphocytes. This result is consistent with early publications suggesting that $A_{2B}$ receptors may play an important role in amplifying the inflammatory responses in the airway. It has been shown that activation of $A_{2B}$ receptors in human mast cells-1 leads to an increase in the release of IL-4 and IL-13 (Ryzhov et al., 2004). IL-4 and IL-13 are well known Th2 cytokines that promote differentiation of Th2 cells and activate B-cells to synthesize and release IgE. In addition, activation of $A_{2B}$ receptors in bronchial epithelial cells leads to the generation of IL-19, which in turn activates monocytes to release tumor necrosis factor α (Zhong et al., 2006). Likewise, activation of $A_{2B}$ receptors in bronchial smooth muscle cells and lung fibroblasts leads to the generations of numerous inflammatory cytokines and chemokines, such as IL-6, monocyte chemotactic protein-1, and IL-8 (Zhong et al., 2004, 2005). It has been shown recently that $A_{2B}$ receptor activation can lead to increase in IL-10 production in lipopolysaccharide-stimulated...
CVT-6883 Inhibits Airway Reactivity and Inflammation


