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A Novel A₁ Adenosine Receptor Antagonist, L-97-1 [3-[2-(4-Aminophenyl)-ethyl]-8-benzyl-7-{2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl}-1-propyl-3,7-dihydro-purine-2,6-dione], Reduces Allergic Responses to House Dust Mite in an Allergic Rabbit Model of Asthma

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Abstract

Adenosine, an important signaling molecule in asthma, produces bronchoconstriction in asthmatics. Adenosine produces bronchoconstriction in allergic rabbits, primates, and humans by activating A₁ adenosine receptors (ARs). Effects of L-97-1 [3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-{2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl}-1-propyl-3,7-dihydro-purine-2,6-dione] a water-soluble, small molecule A₁ AR antagonist were investigated on early and late phase allergic responses (EAR and LAR) in a hyper-responsive rabbit model of asthma. Rabbits were made allergic by intraperitoneal injections of house dust mite [HDM; 312 allergen units (AU)] extract within 24 h of their birth. Booster HDM injections were given weekly for 1 month, biweekly for 4 months, and continued monthly thereafter. Hyper-responsiveness was monitored by measuring lung dynamic compliance (C_{dyn}), after histamine or adenosine aerosol challenge in allergic rabbits. Hyper-responsive rabbits were subjected to aerosol of HDM (2500 AU), 1 h after intragastric administration of L-97-1 (10 mg/kg) solution or an equivalent volume of saline. C_{dyn} was significantly higher after treatment with L-97-1 compared with untreated controls ($p < 0.05$ $n = 5$). Histamine PC₃₀ was significantly higher ($p < 0.05$; $n = 5$) after L-97-1 at 24 h compared with histamine PC₃₀ at 24 h after HDM. Adenosine PC₃₀ was significantly higher at 15 min and 6 h after L-97-1 compared with control ($p < 0.05$; $n = 5$). L-97-1 showed strong affinity for human A₁ ARs in radioligand binding studies and no inhibition toward human phosphodiesterase II, III, IV, and V enzymes. These data suggest that L-97-1 produces a significant reduction of histamine or adenosine-induced hyper-responsiveness and HDM-induced EAR and LAR in allergic rabbits by blocking A₁ ARs and may be beneficial as an oral therapy for human asthma.

ABBREVIATIONS

AR, adenosine receptor; L-97-1, 3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-{2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl}-1-propyl-3,7-dihydro-purine-2,6-dione; HDM, house dust mite; EAR, early allergic response; LAR, late allergic response; AU, allergen unit; C_{dyn}, dynamic compliance; BHR, bronchial hyper-responsiveness; 2-CADO, 2-chloroadenosine; PAEC, pulmonary artery endothelial cell; NMDA, *N*-methyl-D-aspartate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CGS 21680, 2-

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[*p*-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine; ANOVA, analysis of variance; MANOVA, multiple analysis of variance; PDE, phosphodiesterase; ASM, airway smooth muscle

Adenosine is an endogenous nucleoside-signaling molecule and acts on adenosine receptors (ARs) to produce a number of physiological effects in humans, including bronchoconstriction and lung inflammation. Moreover, it is becoming increasingly apparent that adenosine is an important signaling molecule in human asthma. When administered by inhalation, adenosine produces concentration-dependent bronchoconstriction in patients with asthma, but not in normal subjects (Cushley et al., 1983; Polosa, 2002; Rorke and Holgate, 2002). Adenosine levels are increased in the bronchoalveolar fluid of asthmatics and also in the plasma of patients with exercise-induced asthma (Driver et al., 1993; Vizi et al., 2002). There is also an association between allergen exposure and adenosine monophosphate (AMP) responsiveness in asthmatics (Currie et al., 2003). In asthma, the airway response to AMP seems to correlate more closely with disease activity than the response to other more conventional provocative agents, such as methacholine (de Meer et al., 2002).

Adenosine may contribute to the pathogenesis of airway responsiveness and airway inflammation associated with asthma by acting on specific cell surface ARs (Polosa, 2002; Rorke and Holgate, 2002; Livingston et al., 2004). Adenosine receptors belong to the superfamily of receptors known as G protein-coupled receptors. Four adenosine receptor subtypes, A₁, A_{2A}, A_{2B}, and A₃, are expressed in the lung, have been cloned in humans, and have been investigated as potential targets for drug development in asthma (Bjorck et al., 1992; Polosa, 2002; Rorke and Holgate, 2002). By acting through A₁ ARs on a number of different human cell types, adenosine produces bronchoconstriction, inflammation, increased endothelial cell permeability and mucin production, a cardinal feature of airway remodeling, which increase airflow obstruction in asthma (Cronstein et al., 1990, 1992; Salmon et al., 1993; Marquardt, 1997; Wilson and Batra, 2002; McNamara et al., 2004).

Emerging scientific and clinical data support that the A₁ AR is an important AR target in human asthma. Bamifylline is an A₁ AR antagonist approved for the treatment of asthma in Europe (Abbraccio and Cattabeni, 1987; Catena et al., 1988; Morandini, 1988). Theophylline produces its anti-asthma effects in humans with an effective therapeutic plasma level that is less than that required to inhibit human phosphodiesterase enzymes and that would produce antagonism of ARs (Barnes, 2003). Further validation that the A₁ AR is an important target in human asthma is supported by positive results in human asthmatics from early clinical trials with EPI 2010, a respiratory antisense oligonucleotide to the human A₁ AR (Ball et al., 2003).

Previously, it was reported that the allergic rabbit model simulates the human condition of asthma (Metzger et al., 1989; Herd and Page, 1996). Both allergic rabbits and allergic humans behave similarly to airway hyperreactivity to adenosine, histamine, acetylcholine, and platelet-activating factor. After an inhalational challenge, adenosine increases airway reactivity in both allergic humans and allergic rabbits, but not in nonallergic, normal humans, and normal rabbits (Cushley et al., 1983; Ali et al., 1992a, 1994c). With the use of selective pharmacological probes for ARs, the A₁ AR clearly mediates adenosine-induced acute bronchoconstrictor responses in the allergic rabbit model of asthma (Ali et al., 1994c; El-Hashim et al., 1996; Nyce and Metzger, 1997). Moreover, in small airways from allergic rabbits, the expression of the A₁ AR is increased compared with that in small airways from normal rabbits (Ali et al., 1994b).

L-97-1 is a water-soluble small molecule A₁ AR antagonist with high affinity and high selectivity for the human A₁ AR. It is under development as an oral antiasthma treatment for humans. In an allergic rabbit model of asthma, after oral administration, the effect of L-97-1

on house dust mite (HDM) allergen induced early (bronchoconstrictor) and late (inflammatory) allergic responses (EARs and LARs, respectively) as well as histamine and adenosine-induced bronchial hyper-responsiveness were determined.

Materials and Methods

Induction of Allergic Asthma in Rabbits.

Inbred New Zealand White Pasturella-free rabbit littermates were bred and immunized intraperitoneally within 24 h of birth with 312 allergen units (AU) of house dust mite (HDM; Greer Laboratories, Lenoir, NC) suspended in 10% kaolin. The rabbits also received regular rabbit diet and water ad libitum. The injections were repeated weekly for 4 weeks, biweekly for 2 months, and then monthly until the end of the experiment. These rabbits preferentially produce allergen-specific IgE antibody, typically respond to aeroallergen challenge with an early and late phase asthmatic response, and show increased bronchial hyper-responsiveness (Metzger, 1990). Sensitized rabbits show higher titers of HDM-specific IgG (0.505–1.097 U) compared with nonsensitized rabbits (0.062–0.262 U). The rabbits were kept in community cages with 12-h periods of light and dark cycles and were maintained on a standard laboratory rabbit diet with access to water ad libitum. All animal care and experimentation was approved and carried out in accordance with the East Carolina University Institutional Animal Care and Use Committee and in accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Pulmonary Function Measurements.

Four months after birth, the rabbits were screened for adenosine/histamine sensitivity by measuring lung dynamic compliance (C_{dyn}) changes in response to aerosol administration of serial dilutions of adenosine or histamine (0.17–20 mg/ml). Briefly, rabbits were anesthetized and relaxed with 1.5 ml of a mixture of ketamine hydrochloride (35 mg/kg) and acepromazine maleate (1.5 mg/kg) administered intramuscularly. After induction of anesthesia, the rabbits were laid supine on a soft molded animal board in a comfortable position. A salve was applied to the eyes, and they were closed. Each animal was then intubated with a 3.0-mm flexible cuffed Murphy 1 endotracheal tube (Webster Veterinary Supplies, Charlotte, NC) as described previously (Zavala and Rhodes, 1973). A polyethylene catheter of ~2.4 mm o.d. (BD Biosciences, Clay Adams, Parsippany, NJ) with an attached thin-walled latex balloon was passed into the esophagus and maintained at the same distance (~16 cm) from the mouth throughout the experiment. The endotracheal tube was attached to a heated Fleish Pneumotach (size 00; DEM Medical, Richmond, VA) and flow (V) was measured using a Validyn differential pressure transducer (model DP-45-16-1927; Validyn Engineering, Northridge, CA) driven by a Gould carrier amplifier (model 11-4113; Gould Electronics, Cleveland, OH). The esophageal balloon was attached to one side of Validyn differential pressure transducer, and the other side was attached to the outflow of the endotracheal tube to obtain transpulmonary pressure (P_{tp}). Flow was integrated to give a continuous tidal volume. Measurements of total lung resistance (R_t) and dynamic compliance (C_{dyn}) were calculated at isovolumetric and zero flow points. Recording of flow, volume, and pressure were made amplified on an eight-channel Gould 2000W high-frequency amplifier. C_{dyn} was calculated using total volume and the difference in P_{tp} at zero flow. R_t was calculated as the ratio of P_{tp} and V at midtidal lung volumes. These calculations were made automatically with the Buxco automatic pulmonary mechanics respiratory analyzer (Biosystem XA system; Buxco Electronic Inc., Sharon, CT) running on a personal computer, as described previously (Giles et al., 1971). A period of 15 min is allowed after intubation to allow the animals to attain a steady baseline respiration before any procedure.

Measurement of Bronchial Hyper-Responsiveness (BHR).

At 4 months, each of the sensitized rabbits were initially administered histamine or adenosine by aerosol to determine its baseline hyperresponsiveness. Aerosols of either normal saline, histamine, or adenosine were generated by a DeVilbiss nebulizer (DeVilbiss, Somerset, PA) for 2 min at each dose. The ultrasonic nebulizer produced aerosol droplets, of which 80% were $<5\ \mu\text{m}$ in size. Histamine or adenosine aerosol was administered in increasing concentrations (0.17–10 mg/ml) with measurements of pulmonary function for 15 min after 3 min of aerosol for each dose. Animals were not exposed to higher doses of histamine or adenosine after their PC_{30} [concentration (milligrams per milliliter) required to produce 30% reduction in C_{dyn}] was reached. An initial saline aerosol was used to establish baseline. Pulmonary function was summated every 10 breaths of even gradation. Data from spastic breathing were filtered out as artifact. The drug-induced response for each treatment was taken as the lowest consistent C_{dyn} value with 3 min of treatment. This point was typically achieved within the first 2 min after treatment. Allergic rabbits that do not attain a PC_{30} to histamine or adenosine above 20 and 10 mg/ml, respectively, were excluded from the study. Less than 1% of all sensitized rabbits did not attain this PC_{30} to histamine or adenosine.

Effect of L-97-1 on Allergen-Induced EAR and LAR.

Sensitized rabbits exposed to allergen aerosol are susceptible to an allergic response characterized by a phasic bronchoconstriction (EAR) and airway inflammation (LAR). The following procedure was designed to investigate the effect of L-97-1 on HDM allergen-induced EAR and LAR in the allergic rabbit model. Rabbits that had not been used for any airway provoking procedure were anesthetized and intubated as described above. After a steady baseline respiration is attained, the animals were aerosolized with 2500 AU of HDM allergen for about 10 min or until the allergen was exhausted. Pulmonary function (C_{dyn}) was then measured at 15-min intervals during the next 6 h to determine the effect of HDM allergen on early (0–60 min) and late (120–360 min) allergic responses (EAR and LAR, respectively). The same procedure was repeated with L-97-1 (10 mg/kg) oral gavage administered 1 h before allergen challenge in the same animals after at least a 2-week washout period. Anesthesia was maintained with a mixture of ketamine/acepromazine (80:20) at a dose of 0.15 mg/kg given every 45 min to 1 h.

Effect of L-97-1 on Allergen-Induced BHR to Histamine.

To determine the effect of L-97-1 on BHR, the following protocol was used. Allergic rabbits that had not been aerosolized with allergen, histamine, or adenosine for at least 2 weeks ($n = 5$) were aerosolized with histamine as described above to determine their baseline PC_{30} to histamine and BHR to histamine. Twenty-four hours later, the rabbits were given an aerosol challenge of 2500 AU of HDM allergen. Then, BHR measurement with histamine aerosolized challenge was repeated at 24 h after allergen challenge. The same protocol was employed to test the effectiveness of L-97-1 (10 mg/kg) administered as an oral gavage 1 h before HDM allergen challenge on BHR to histamine.

Effect of L-97-1 on BHR to Adenosine.

To determine the effect of L-97-1 on BHR to adenosine, the following protocol was used. Allergic rabbits that had not been aerosolized with allergen, histamine, or adenosine for at least 2 weeks ($n = 5$) were aerosolized with adenosine as described above to determine their baseline PC_{30} to adenosine. The next day, 1 h after oral administration of L-97-1 (10 mg/kg), measurements of BHR to adenosine were again taken at 15 min, 6 h, and 24 h in the same rabbits.

Plasma Levels of L-97-1.

An ear artery sample of blood was collected in tubes containing EDTA at 0 min, 15 min, 30 min, 1 h, 2 h, 3 h, 6 h, 8 h, and 24 h after L-97-1 administration. The samples were centrifuged at 5000g for 5 min, and plasma was collected and frozen at -20°C until used. Serum levels of L-97-1 were measured by electrospray liquid chromatography/tandem mass spectrometry method validated at Prevalere Life Sciences, Inc. (Whitesboro, NY).

Effect of L-97-1 on Contractile Responses in *in Vitro* Muscle Tension Studies in Small Airways of Allergic Rabbits.

Allergic rabbits were euthanized with sodium pentobarbital (100 mg/kg i.v) in accordance with the guidelines of the Animal Use and Care Committee of the Brody School of Medicine (East Carolina University). Lungs were removed and immediately placed in oxygenated, ice-cold Krebs-Henseleit buffer, pH 7.4. Secondary (5 mm) and tertiary (2–4 mm) airways were dissected out of the lung tissue. During the dissection, tissue was immersed in ice-cold oxygenated buffer. Bronchioles were cut into small rings and mounted in 10-ml organ baths with stainless steel hooks and surgical thread (000) with a resting tension of 500 mg. Organ baths contained oxygenated and heated (37°C) Krebs-Henseleit buffer. Bronchiole rings were equilibrated with the organ bath environment for 2 h, with a complete change of buffer every 15 min. Contractions of each bronchiole ring were expressed as a percentage of the force measured when rings were treated with 50 mM KCl. Isometric tension was measured by force displacement transducers (BIOPAC Systems Inc., Santa Barbara, CA) connected to BIOPAC MP100 data acquisition and analysis hardware from BIOPAC Systems Inc. After bronchioles were stimulated with KCl and the buffer in each organ bath was exchanged three times in rapid succession, tissues were given a 30 min recovery period before challenge with the increasing concentrations of the nonselective AR agonist 2-chloroadenosine (2-CADO) (5×10^{-5} and 10^{-4} M). After washing out the agonist from the organ baths, the bronchioles were given a 30-min period to return to baseline tension before a 30-min treatment with L-97-1 at a concentration of 10^{-6} or 10^{-5} M. Bronchioles were again challenged with the same concentrations of 2-CADO. This protocol was repeated on the bronchioles using a single dose of histamine (5×10^{-6} M) before and after treatment with L-97-1. Each experimental condition was performed in bronchioles from three different rabbits in replicates of 8.

Radioligand Binding Assays.

To determine the affinity for L-97-1 for the human A_1 AR, A_{2A} AR, and A_{2B} ARs, the following protein sources were used: membranes from human pulmonary artery endothelial cells (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) expressing the human A_1 AR and membranes from human embryonic kidney 293 cells expressing the recombinant human A_{2A} and human A_{2B} ARs (Receptor Biology, Inc., Beltsville, MD).

Human Pulmonary Artery Endothelial Cells (PAECs): Culture and Membrane Preparation.

Human PAECs were obtained from Cambrex Bio Science Walkersville, Inc., and grown in a multilayer tissue culture vessel for membrane preparation in an atmosphere of 95% O_2 and 5% CO_2 . The cells were grown and maintained in medium recommended by the manufacturer, EMB-2 (Cambrex Bio Science Walkersville, Inc.) which contains 2.0% fetal bovine serum. The cells were washed three times with phosphate-buffered saline and then suspended in lysis buffer (10 mM Tris HCl, pH 7.4, containing 5 mM EDTA, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 10 $\mu\text{g}/\text{ml}$ benzamidine, and 2 $\mu\text{g}/\text{ml}$ pepstatin). The cells were homogenized by sonication. The homogenate was centrifuged at 1000g at 4°C for 10 min. The supernatant was centrifuged at 30,000g for 45 min. The pellet was reconstituted in reconstitution buffer (50 mM Tris HCl, pH 7.4, 5 mM EDTA, 10 mM MgCl_2 , 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 10 $\mu\text{g}/\text{ml}$

benzamidine, and 2 µg/ml pepstatin). The protein content was determined by Bradford reagent using bovine serum albumin as standard. The aliquots were stored at -80°C until used.

Determination of the Affinity of L-97-1 for Human A₁ and Recombinant Human A_{2A}, and A_{2B} Adenosine Receptors in Inhibition, Competition Radioligand Binding Assays.

Radioligand competition binding experiments were performed with membranes from human PAECs or human embryonic kidney-transfected cells in a total volume of 0.2 ml in incubation buffer at room temperature with the selective adenosine receptor antagonist radioligands and under the conditions as determined or recommended by the supplier of the membranes that are presented in Table 1. After incubation, the samples were filtered rapidly under vacuum through polyethyleneimine-treated filters and washed four times with 3 ml of ice-cold buffer using a cell harvester (Skatron, Lier, Norway). The filters were dried and counted for radioactivity with the use of a liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Two to four experiments were performed and assayed in duplicate.

The affinity of L-97-1 for the recombinant human A₃ AR was determined in another laboratory (Dr. Gary L. Stiles, Duke University Medical Center, Durham, NC) in Chinese hamster ovary cells expressing the recombinant human A₃ AR with the use of N⁶-(4-amino-3-[¹²⁵I]iodobenzyl)adenosine-5'-N-methylcarboxamide, as the competing radioligand in competition radioligand binding assays (*n* = 2).

To further validate the selectivity of L-97-1 for the human A₁ AR, the affinity of L-97-1 for a number of other receptors was determined by NovaScreen (Hanover, MD) with the use of radioligand competition binding assay protocols similar to that described above and selective radioligands for the following receptors. L-97-1 (10 µM) was tested in competition radioligand binding assays for the rat adrenergic, α₁ and α₂, peripheral benzodiazepine, nonselective dopamine, glutamate [α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, kainate, NMDA agonist, and glycine NMDA sites), strychnine-sensitive glycine, H₁ histamine, H₂ histamine, and H₃ histamine, non-selective central muscarinic, nonselective peripheral muscarinic, nonselective serotonin, and nonselective opiate receptors.

Assay for Inhibition of Human Phosphodiesterase Enzymes.

Assays for inhibition of human phosphodiesterase enzymes II, III, IV, and V, were determined by Cerep (Celle l'Evescault, France). Test compounds, L-97-1 (100 µM) and theophylline (100 µM), were tested in duplicate. In each experiment, the respective reference compound was tested at a minimum of seven concentrations in duplicate to obtain an inhibition curve to validate the experiment. Radioactivity was determined with a scintillation counter (TopCount; PerkinElmer Life and Analytical Sciences). General procedures and experimental conditions are given in Tables 2 and 3, respectively.

Chemicals.

[³H]DPCPX and [³H] CGS 21680 were purchased from PerkinElmer Life and Analytical Sciences. (*R*)-N⁶-Phenylisopropyladenosine, 2-chloro-N⁶-cyclopentyladenosine, 5'-N-ethylcarboxamidoadenosine, DPCPX, and all other common reagents of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO). L-97-1 was custom synthesized by ChemSyn Laboratories (Lenexa, KS) and provided by Constance N. Wilson (Endacea, Inc., Research Triangle Park, NC).

Statistical Analysis.

To assess the effect of L-97-1 on the changes in pulmonary function after adenosine, histamine, and allergen challenge, the area under the curve in square centimeters is digitized by computer-

assisted plethrometry for each rabbit during a 6-h period after adenosine and allergen challenge and at 24 h after adenosine, histamine, and allergen challenge. The early and late phase responses are determined at 0 to 1 h and 1 to 6 h, respectively, as previously established in this allergic rabbit model. The percentage change in C_{dyn} is calculated for each time point for allergen challenge (every 15 min for 6 h). Statistical significance in time series between the control and drug-treated groups were determined by two-way multiple ANOVA (MANOVA). Comparisons between control and test values at the same time point were determined by post hoc comparison of two means. Airway hyper-responsiveness for adenosine and histamine is calculated by determining the concentration of adenosine or histamine (milligrams per milliliter) required to reduce the C_{dyn} by 30% from baseline (PC₃₀). Significance for histamine responses is determined by comparing these values using ANOVA with post hoc least square difference determination between values and for adenosine responses using Kruskal-Wallis test. In the in vitro muscle tension studies statistical significance of the results was determined using the Student's *t* test for paired data. Results are expressed as mean ± S.E.M. A value of *p* < 0.05 is considered significant. Radioligand binding data were analyzed by nonlinear regression using a sigmoidal dose-response curve with variable slope (Prism version 3.0; GraphPad Software Inc., San Diego, CA).

Results from assays for inhibition of human phosphodiesterase enzymes are expressed as a percentage of inhibition of control values obtained in the presence of the test compounds. IC₅₀ values (concentration causing a half-maximal inhibition of control values) and Hill coefficients (*n_H*) were determined for the reference compounds by nonlinear regression analysis of their inhibition curves. These parameters were obtained by Hill equation curve fitting. The IC₅₀ values obtained for the reference compounds have passed the required inspections. They are within accepted limits of historic averages obtained ± 0.5 log units.

Results

Effect of L-97-1 on EAR and LAR.

Figure 1 shows the effect of L-97-1 (10 mg/kg oral) administration on HDM allergen-induced EAR and LAR. L-97-1-treated rabbits showed significantly higher C_{dyn}, up to 6 h compared with the untreated control group (*n* = 5). The curve is significant at all time points after 30 min (MANOVA; *p* < 0.05).

Effect of L-97-1 on BHR to Histamine.

Figure 2 shows the effect of L-97-1 (10 mg/kg oral) administration on HDM-induced bronchial hyper-responsiveness to histamine in the allergic rabbits. Allergen challenge significantly reduced PC₃₀ (histamine) to 4 ± 1 mg/ml 24 h after allergen challenge from the baseline PC₃₀ of 15 ± 4 mg/ml (no allergen, no drug) (*p* < 0.05; *n* = 5). Administration of L-97-1 (10 mg/kg oral), 1 h before allergen challenge markedly increased the PC₃₀ from 4 ± 1 mg/ml (24 h after allergen challenge with no drug) to 20 ± 8 mg/ml (24 h after allergen challenge plus L-97-1) (*p* < 0.05; *n* = 5).

Effect of L-97-1 on BHR to Adenosine.

Figure 3 shows the effect of L-97-1 (10 mg/kg oral) administration on bronchial hyper-responsiveness to adenosine in allergic rabbits. PC₃₀ adenosine increased significantly from baseline 4.14 ± 0.83 to 38.33 ± 1.67 mg/ml (*p* < 0.05; *n* = 5), 15 min after the single administration of L-97-1 (10 mg/kg administered as oral gavage 1 h before adenosine challenge). The PC₃₀ after 6 h (11.67 ± 4.41 mg/ml) and 24 h (5.83 ± 2.2 mg/ml) of single administration of L-97-1 (10 mg/kg oral) was higher compared with baseline reaching statistical significance at 6 h compared with the baseline (*p* < 0.05; *n* = 5).

Plasma Levels of L-97-1.

Table 4 shows the levels of L-97-1 in a 24-h period after a single oral administration of L-97-1 (10 mg/kg). The effect of L-97-1 on LAR and adenosine-induced bronchial hyper-responsiveness at 6 h after administration of L-97-1 (10 mg/kg) correlates with a plasma level of 13 ng/ml. L-97-1 blocked bronchial hyper-responsiveness significantly increasing the PC₃₀ histamine at 24 h after allergen challenge. This lasting effect of L-97-1 at 24 h suggests that a plasma level of 3 ng/ml is an effective plasma concentration.

Effect of L-97-1 on Contractile Responses in in Vitro Muscle Tension Studies in Small Airways of Allergic Rabbits.

Figure 4 shows that in in vitro muscle tension pharmacology studies in small airways from allergic rabbits, L-97-1 (10^{-5} and 10^{-6} M) selectively blocks the contractile responses of 2-CADO (5×10^{-5} and 10^{-4} M) ($p \leq 0.006$) in a concentration-dependent manner without blocking those of histamine (5×10^{-6} M).

Radioligand Binding Studies.

Affinities of L-97-1 and other reference adenosine receptor ligands to human A₁, A_{2A}, and A_{2B} ARs are presented in Table 5. L-97-1 has high affinity (580 nM) and high selectivity for the human A₁ AR with no binding to recombinant human A_{2A} or A_{2B} ARs at high concentration (≤ 100 μ M). Moreover no binding was demonstrated for L-97-1 (≤ 1 mM) to the recombinant human A₃ AR (data not shown). There was no binding of L-97-1 to rat adrenergic, α_1 and α_2 , peripheral benzodiazepine, non-selective dopamine, glutamate (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, kainate, NMDA agonist, and glycine NMDA sites), strychnine-sensitive glycine, H₁ histamine, H₂ histamine, and H₃ histamine, nonselective central muscarinic, nonselective peripheral muscarinic, nonselective serotonin, and nonselective opiate receptors (data not shown).

Phosphodiesterase Inhibition Assays.

The effects of L-97-1 (100 μ M) and theophylline (100 μ M) on the human phosphodiesterase II, III, IV, and V, enzymes are summarized in Table 6 where the IC₅₀ values for the reference compounds are also indicated. As opposed to theophylline (100 μ M), which inhibits human PDE enzymes II, III, and IV at 19, 28, and 21% inhibition, respectively, L-97-1 (100 μ M) does not inhibit human PDE enzymes II, III, IV, or V.

Discussion

In the present study, a rabbit model of allergic asthma was used to study the effects of a novel A₁AR antagonist, L-97-1, on HDM-induced early and late phase allergic responses and BHR to histamine and adenosine. In this proof of concept study, administration of a high dose for L-97-1 (10 mg/kg) given orally to allergic rabbits significantly improved both EAR and LAR responses and increased lung compliance (C_{dyn}) after HDM allergen challenge. Pretreatment with this same dose of L-97-1 also blocked BHR to both adenosine and histamine in allergic rabbits. Pharmacokinetic profile of L-97-1 in plasma shows that L-97-1 produced these anti-asthma effects in allergic rabbits at plasma levels (3–13 ng/ml; 10 nM) that do not inhibit human phosphodiesterase II, III, IV, or V enzymes. Radioligand binding studies in human ARs and other receptors confirm that this compound has high affinity and high selectivity for the human A₁ AR. Moreover, in vitro pharmacology studies in small airways of allergic rabbits suggest that L-97-1 selectively produces its antiasthma effects in allergic rabbits by blocking ARs.

The endogenous nucleoside, adenosine is reported to produce acute bronchoconstriction through indirect effects by inducing the release of preformed and newly formed mediators from

mast cells and possibly direct effects on airway smooth muscle (ASM) and adrenergic nerve endings (Polosa, 2002; Livingston et al., 2004). Previously, it is reported that A₁ ARs on ASM are a direct target for adenosine in humans and allergic rabbits (Ali et al., 1994b; Nyce and Metzger, 1997; Mundell et al., 2001). Moreover, in the allergic rabbit model of asthma, administration of a respiratory antisense oligodeoxynucleotide specific for the A₁ AR, EPI 2010, reduced the density of A₁ ARs on ASM and attenuated adenosine-induced acute bronchoconstriction (Nyce and Metzger, 1997). In the present study, PC₃₀ for adenosine is increased significantly at 15 min and 6 h after oral administration of L-97-1. In previous studies, it was reported that BHR to adenosine in allergic rabbit model of asthma is mainly due to up-regulation of A₁ ARs on ASM (Ali et al., 1994b; Nyce and Metzger, 1997). Thus, in the present study, L-97-1 may block the BHR to adenosine in allergic rabbits due to the antagonistic action by this A₁ AR antagonist on A₁ ARs on ASM.

Airway hyper-responsiveness to allergen is considered to be a hallmark of allergic asthma. The rabbit model of allergic asthma has been previously used to test antiasthma drugs that are in current use for treatment of human asthma (Ali et al., 1992a,c, 1994a). Both theophylline and beclomethasone administered as inhalational treatments inhibit EAR and LAR responses in this rabbit model of allergic asthma (Ali et al., 1992c, 1994a). Both allergic rabbits and allergic humans share many common features of asthma. These features include airway hyperreactivity to adenosine, histamine, acetylcholine, platelet-activating factor, development of inflammation and permeability changes in the airways, release of mediators, including neutrophil and eosinophil chemotactic factors, production of antigen-specific IgE antibodies, and mast cell dependence (Larsen et al., 1984; Metzger et al., 1989; Herd and Page, 1996; Gozzard et al., 1997; Gascoigne et al., 2003). Moreover, adenosine levels are increased in the bronchoalveolar fluid of both humans and rabbits with allergic asthma (Ali et al., 1992b; Driver et al., 1993).

We therefore investigated the effect of L-97-1, an A₁ AR antagonist in development as an oral treatment for asthma in humans, on HDM allergen-induced EAR and LAR as well as histamine-induced increases in BHR 24 h after allergen challenge, in the allergic rabbit model of asthma. L-97-1 (10 mg/kg) administered 1 h before HDM allergen administration significantly increased C_{dyn} at all time points after 30 min up to 6 h, thus preventing the decline in C_{dyn} and blocking both EAR and LAR responses after allergen challenge in rabbits with allergic asthma. The increase in C_{dyn} during the EAR response can be explained by the direct blocking of A₁ ARs on airway smooth muscle as some of the previous studies have suggested (Ali et al., 1994b; Nyce and Metzger, 1997). Previously, it was reported that EPI 2010 reduces EAR response in allergic rabbits and decreases the expression of A₁ ARs in ASM in allergic rabbits (Nyce and Metzger, 1997). Moreover, theophylline inhibits EAR and LAR responses in the allergic rabbit model at a dose that is lower than that which would produce plasma levels to inhibit PDE enzymes (Ali et al., 1992c). The inhibitory activity of L-97-1 on A₁ ARs cannot be explained by the inhibition of PDE class of enzymes, since the plasma levels of L-97-1 detected after oral administration of this compound are too low to cause any inhibition of PDE enzymes. Together, the results of these studies suggest that L-97-1 may block allergen-induced EAR responses by blocking activation of A₁ ARs by adenosine present in bronchoalveolar lavage fluid of allergen-challenged rabbits. This effect of L-97-1 on EAR response in allergic rabbits may also be, in part, a function of its antagonistic effect on the release of preformed or newly formed mediators from mast cells or adrenergic nerve terminals. A₁ ARs may be up-regulated in human mast cells which are immunologically sensitized by IgE (Peachell et al., 1988). The effect of L-97-1 on the release of preformed or newly formed mediators, i.e., histamine and leukotrienes, from immunologically sensitized mast cells, are studies for future investigations.

The bronchoconstrictor effect of adenosine in the asthmatic lung is mediated through its specific cell surface receptors: 1) the effects of adenosine are not reproduced by inosine, the

deaminated metabolite of adenosine, or a closely related purine nucleoside, guanosine; however, adenosine mono- and diphosphates, i.e., AMP and ADP (which are rapidly converted to adenosine in the lung under physiological conditions), are equipotent with adenosine as bronchoconstrictor agents (Mann et al., 1983); 2) theophylline and bamifylline preferentially produce their antiasthma effects in humans at concentrations that do not inhibit phosphodiesterase enzymes (Foutillan et al., 1983; Ginesu et al., 1987; Catena et al., 1988; Clarke et al., 1989; Spoto et al., 1995); and 3) dipyridamole, an uptake blocker of adenosine into the cells, enhances adenosine-induced bronchoconstriction in asthmatic patients (Cushley et al., 1986).

In humans bamifylline produces its antiasthma effects by selectively blocking A₁ ARs. Bamifylline binds to the human A₁ AR and human A_{2A} AR with higher affinity for the human A₁ AR (1.93 μM) than the human A_{2a} AR (12.9 μM) and does not bind to human A_{2B} or human A₃ ARs (≤100 μM; Constance N. Wilson, personal communication). The therapeutic plasma concentration of bamifylline is 500 times less than that needed to inhibit human II, III, IV, and V PDE enzymes (Ginesu et al., 1987; Mann et al., 1983; Constance N. Wilson, personal communication). Moreover, in humans theophylline produces its antiasthma effects most likely by blocking ARs. Theophylline binds nonselectively to all the human AR subtypes (Klotz et al., 1998). The effective therapeutic plasma concentrations for theophylline in humans is 10 to 100 times below that needed to inhibit human PDE enzymes *in vitro* (Clarke et al., 1989). Compared with bamifylline and theophylline, L-97-1 binds selectively to the human A₁ AR. Moreover, L-97-1 produces its antiasthma effects in allergic rabbits at concentrations that are 1000 to 10,000 times less than that needed to inhibit human PDE enzymes.

Together, these data suggest that L-97-1 is a potent, selective antagonist of A₁ ARs and its efficacy in inhibiting the allergen-induced EAR, LAR responses and attenuating BHR to histamine after allergen challenge and adenosine-induced BHR in allergic asthmatic rabbits is most likely related to its antagonism of A₁ ARs on ASM and perhaps on mast cells, as well as other cell types, such as inflammatory cell types that play an important role in the LAR response. Activation of A₁ ARs on human neutrophils and macrophages has been shown to produce proinflammatory effects (Cronstein et al., 1990, 1992; Salmon et al., 1993). The effect of L-97-1 on airway inflammation in the allergic rabbit model of asthma is an area for future investigation.

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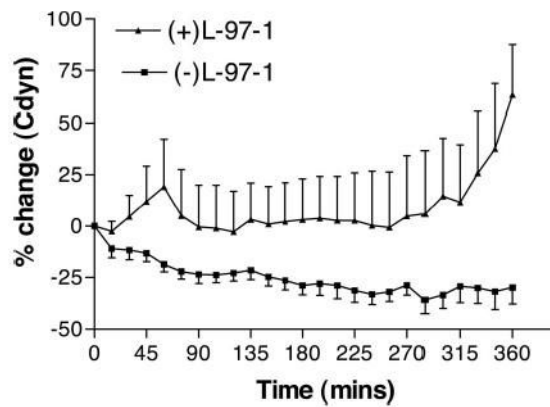


Fig. 1. Lung Cdyn in HDM-challenged rabbits with (+) and without (-) L-97-1 (10 mg/kg) treatment. HDM was administered to sensitized rabbits by aerosolization (2500 AU). L-97-1 was administered by intragastric tube 1 h before HDM administration. Ventilation was monitored in the anesthetized rabbits up to 6 h after HDM administration. The curve is significant at all time points greater than 30 min, after treatment with L-97-1 ($p < 0.05$; MANOVA). Data are expressed as mean \pm S.E.M. ($n = 5$).

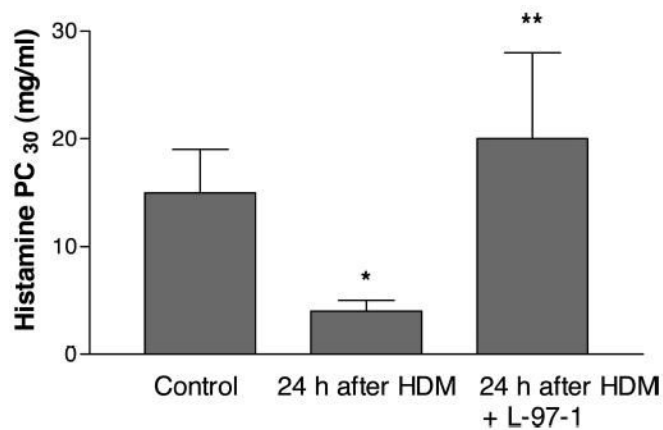


Fig. 2. Histamine responses [histamine PC₃₀ (milligrams per milliliter)] in rabbits aerosolized with HDM allergen with or without L-97-1 (10 mg/kg) administered by intragastric tube 1 h before HDM administration. Control measurement PC₃₀ histamine was taken without HDM and without prior drug treatment. PC₃₀ histamine was measured again 24 h after HDM or HDM + L-97-1 treatment. Data are expressed as mean \pm S.E.M. ($n = 5$). *, $p < 0.05$ compared with control; **, $p < 0.05$ compared with 24 h after HDM treatment (ANOVA).

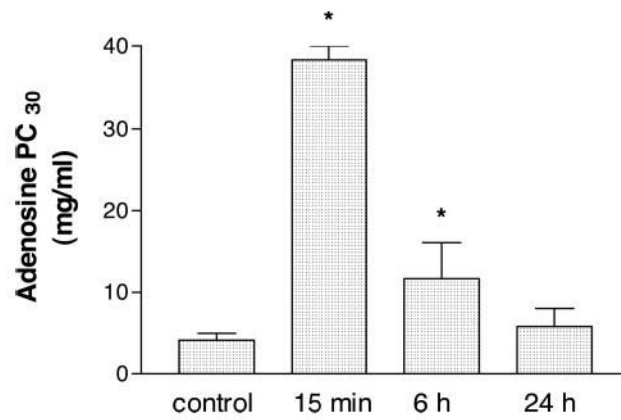


Fig. 3.

Adenosine responses (adenosine PC₃₀ milligrams per milliliter) in allergic rabbits with or without L-97-1. Adenosine PC₃₀ measurements were taken 15 min, 6 h, and 24 h after administration of L-97-1 (10 mg/kg) by intragastric tube. Control measurements to adenosine in allergic rabbits were taken without HDM allergen challenge and 24 h before L-97-1 administration. Data are expressed as mean \pm S.E.M. ($n = 5$). *, $p < 0.05$ compared with controls (Kruskal-Wallis test).

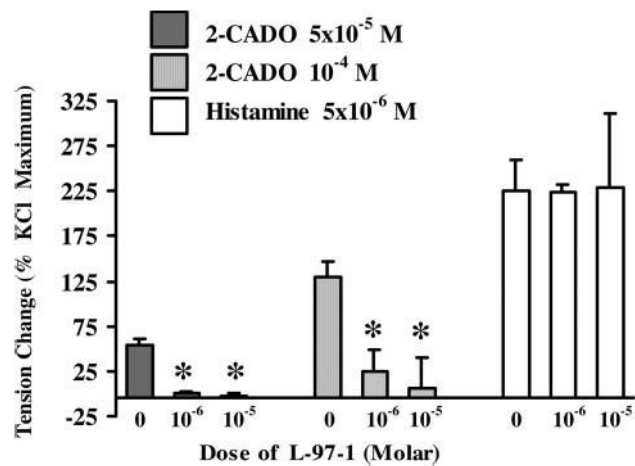


Fig. 4.

Contractile responses to 2-chloroadenosine and histamine in in vitro muscle tension studies of small airways from allergic rabbits. The percentage of contraction by 2-CADO (5×10^{-5} and 10^{-4} M) and histamine (5×10^{-6} M) was calculated by comparing the response of each bronchiole to the tension produced when activated with 50 mM KCl. Each experimental condition was performed in bronchioles from three different rabbits in replicates of eight. Data are expressed as mean \pm S.E.M. *, $p < 0.006$ compared with agonist without L-97-1 (Student's t test for paired data).

TABLE 1

Assay table for determination of the affinity of L-97-1 for human A₁ and recombinant human A_{2A} and A_{2B} adenosine receptors in inhibition, competition radioligand binding assays

Concentration of [³H]DPCPX used in A₁ AR radioligand competition binding assay was determined from saturation radioligand binding assays in membranes from human PAECs to determine the K_d (48 nM; average of two experiments performed in duplicate). Adenosine deaminase, [³H]DPCPX, [³H]CGS 21680 (a selective radioligand for the A_{2A} AR), N⁶-R-phenylisopropyladenosine, and 5'-(N-ethylcarboxamido)-adenosine were used to determine nonspecific binding for the A₁ AR and A_{2A} and A_{2B} ARs, respectively.

	Human A ₁ AR	Recombinant Human A _{2A} AR	Recombinant Human A _{2B} AR
Protein source/total amount	Human PAECs expressing huA ₁ AR/10 µg	HEK-293 cells expressing rehuA _{2A} AR/7.4 µg	HEK-293 cells expressing rehuA _{2B} AR/21.2 µg
Washing buffer	50 mM Tris; pH 7.4	50 mM Tris; pH 7.4	50 mM Tris; pH 7.4
Incubation buffer	50 mM Tris; pH 7.4; 0.2 U/ml AD	50 mM Tris; pH 7.4; 2.0 U/ml AD; 10 mM MgCl ₂ ; 1 mM EDTA	50 mM Tris; pH 7.4; 2.0 U/ml AD; 10 mM MgCl ₂ ; 1 mM EDTA; 0.1 M benzamidine
Radioligand/concentration	[³ H]DPCPX/48 nM	[³ H]CGS 21680/21 nM	[³ H]DPCPX/34 nM
Nonspecific binding/concentration	R-PIA/100 µM	NECA/50 µM	NECA/100 µM
Incubation time	2 h	90 min	60 min
Filter type	GF/C	934/AH	GF/C

hu, human; rehu, recombinant human; AD, adenosine deaminase; R-PIA, N⁶-R-phenylisopropyladenosine; NECA, 5'-(N-ethylcarboxamido)-adenosine.

TABLE 2
Assay table for inhibition of human phosphodiesterase enzymes
The assays were performed using the following general procedures.

Assay	Origin	Reference Compound
Phosphodiesterase II (<i>h</i>)	Differentiated U-937 cells	EHNA
Phosphodiesterase III (<i>h</i>)	Human platelets	Milrinone
Phosphodiesterase IV (<i>h</i>)	U-937 cells	Rolipram
Phosphodiesterase V (<i>h</i>)	Human platelets	Dipyridamole

h, human; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine.

TABLE 3**Assay table for inhibition of human phosphodiesterase enzymes**

The experimental conditions are summarized below.

Assay	Substrate	Incubation	Reaction Product	Method of Detection
Phosphodiesterase II (<i>h</i>)	[³ H]cAMP (1 μM)	30 min/30°C	[³ H]5'AMP	Liquid scintillation
Phosphodiesterase III (<i>h</i>)	[³ H]cAMP (0.1 μM)	30 min/30°C	[³ H]5'AMP	Liquid scintillation
Phosphodiesterase IV (<i>h</i>)	[³ H]cAMP (1 μM)	30 min/30°C	[³ H]5'AMP	Liquid scintillation
Phosphodiesterase V (<i>h</i>)	[³ H]cGMP (1 μM)	30 min/30°C	[³ H]5'GMP	Liquid scintillation

h, human.

TABLE 4

Plasma levels (nanograms per milliliter) of L-97-1 in a 24-h period after oral administration of L-97-1 (10 mg/kg)

Data are presented as mean \pm S.E.M., $n = 5$.

Sample	15 min	30 min	1 h	2 h	3 h	6 h	8 h	24 h
Mean	140.70	116.18	73.71	23.65	20.68	12.76	13.25	3.17
S.E.M.	73.04	58.42	37.60	9.40	6.45	4.79	3.81	0.43

TABLE 5

Affinities of L-97-1 for human A₁, recombinant human A_{2A}, and A_{2B} adenosine receptors

The following compounds are used as competing radioligands for the A₁ and A_{2A} and A_{2B} ARs: [³H]DPCPX (A₁ and A_{2B} ARs) and [³H]CGS 21680 (A_{2A} ARs). The following compounds are used as reference compounds: DPCPX and 2-chloro-N⁶-cyclopentyladenosine (CCPA) are reference compounds for the A₁ AR; DPCPX is also a reference compound for the A_{2B} AR; CGS 21680 is a reference compound for the A_{2A} AR. The studies presented in this table are inhibition competition radioligand binding assays to determine the IC₅₀ or K_i (measurements of affinity) of the ligand for the AR. The K_i or IC₅₀ are inversely related to the affinity of the ligand for the receptor; i.e. the lower the K_i or IC₅₀, the higher the affinity.

Ligand	Human A ₁ ([³ H]DPCPX)			Human A _{2A} ([³ H]CGS 21680)			Human A _{2B} ([³ H]DPCPX)		
	IC ₅₀	K _i	<i>n</i>	IC ₅₀	K _i	<i>n</i>	IC ₅₀	K _i	<i>n</i>
L-97-1	1.42 ± 0.57	0.58 ± 0.33	4	>100	N.D.	3	>100	N.D.	3
DPCPX	0.08 ± 0.04	0.04 ± 0.02	4	N.D.	N.D.		0.22 ± 0.07	0.12 ± 0.04	3
CCPA	0.03 ± 0.02	0.02 ± 0.01	2	N.D.	N.D.		N.D.	N.D.	3
CGS 21680	N.D.	N.D.		0.32 ± 0.22	0.16 ± 0.11	3	N.D.	N.D.	

N.D., not determined.

TABLE 6**Effects of L-97-1 and theophylline on human PDE enzymes and IC₅₀ values for the reference compounds**

For the test compounds, the results are expressed as a percentage of inhibition of control activity (mean values; $n = 2$).

Assay	L-97-1	Theophylline	Reference Compound		
			IC ₅₀	n_H	
		100 μM		μM	
PDE II (<i>h</i>)	—	19	EHNA	4.7	0.7
PDE III (<i>h</i>)	—	28	Milrinone	0.39	0.8
PDE IV (<i>h</i>)	—	21	Rolipram	0.21	0.6
PDE V (<i>h</i>)	—	—	Dipyridamole	0.80	1.2

h, human; —, inhibition of less than 10%; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine.