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Title Page

A Novel A_1 Adenosine Receptor Antagonist (L-97-1) Reduces Allergic Responses to House Dust Mite in an Allergic Rabbit Model of Asthma

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Running Title Page

Running Head: L-97-1 reduces allergic responses in asthma

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Non-standard abbreviations: AR: adenosine receptor, AMP: adenosine monophosphate, ASM: airway smooth muscle, BHR: bronchial hyper-responsiveness, Cdyn: dynamic compliance, EAR: early allergic response, HDM: house dust mite, LAR: late allergic response, PDE: phosphodiestease

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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 A_1 adenosine receptors (AR). Effects of L-97-1, a water soluble, small molecule A_1 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 AU) extract within 24 h of their birth. Booster HDM injections were given weekly for one month, biweekly for 4 months and continued monthly thereafter. Hyper-responsivenessness was monitored by measuring lung dynamic compliance (Cdyn), following 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 (10mg/kg) solution or an equivalent volume of saline. Cdyn was significantly higher following treatment with L-97-1 compared to untreated controls ($p<0.05$, n=5). Histamine PC_{30} was significantly higher ($p<0.05$, n=5) following L-97-1 at 24 h compared to histamine PC_{30} at 24 h following HDM. Adenosine PC_{30} was significantly higher at 15 min and 6 h following L-97-1 compared to control ($p<0.05$, n=5). L-97-1 showed strong affinity for human A_1 ARs in radioligand binding studies and no inhibition towards 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-responsive and HDM-induced EAR and LAR in rabbits by blocking A1 ARs and may be beneficial as an oral therapy for human asthma.

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Introduction

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 metacholine (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 (Livingston et al., 2004; Polosa, 2002; Rorke and Holgate , 2002). Adenosine receptors belong to the superfamily of receptors known as G protein coupled receptors. Four adenosine receptor subtypes, $A_{1,}$ $A_{2A,}$ A_{2B} and A_3 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 A_1 ARs on a number of different human cell types, adenosine produces

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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; Cronstein et al., 1992; Marquardt, 1997; McNamara et al., 2004; Salmon et al., 1993; Wilson and Batra, 2002).

Emerging scientific and clinical data support that the A_1 AR is an important AR target in human asthma. Bamiphylline is an A_1 AR antagonist approved for the treatment of asthma in Europe (Abbrachio 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 A_1 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 A_1 AR (Ball et al., 2003).

Previously it was reported that the allergic rabbit model simulates the human condition of asthma (Herd and Page, 1996; Metzger et al., 1989). Both allergic rabbits and allergic humans behave similarly to airway hyperreactivity to adenosine, histamine, acetylcholine, and platelet activating factor. Following an inhalational challenge, adenosine increases airway reactivity in both allergic humans and allergic rabbits, but not in non-allergic, normal humans and normal rabbits (Ali et al., 1992 a; Ali et al., 1994 a; Cushley et al., 1983). With the use of selective pharmacological probes for ARs, the A_1 AR clearly mediates adenosine-induced acute bronchoconstrictor responses in the allergic rabbit model of asthma (Ali et al., 1994 a; El-Hashim et al., 1996; Nyce and Metzger,

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1997). Moreover, in small airways from allergic rabbits, the expression of the A_1 AR is increased compared to that in small airways from normal rabbits (Ali et al., 1994 b). .

L-97-1 is a water-soluble small molecule A_1 AR antagonist with high affinity and high selectivity for the human A_1 AR. It is under development as an oral anti-asthma treatment for humans. In an allergic rabbit model of asthma, following oral administration, the effect of L-97-1 on House Dust Mite (HDM) allergen induced early (bronchoconstrictor) and late (inflammatory) allergic responses (EAR, and LAR, respectively), as well as histamine and adenosine-induced bronchial hyper-responsiveness were determined.

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Materials and Methods

Induction of Allergic Asthma in Rabbits

Inbred New Zealand White Pasturella-free rabbit littermates were bred and immunized intraperitoneally within 24 hours of birth with 312 Allergen Units 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 – 1.097U) compared to non-sensitized rabbits (0.062 – 0.262U). The rabbits were kept in community cages with 12-hour 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 (Cdyn)

Four months after birth, the rabbits were screened for adenosine/histamine sensitivity by measuring lung dynamic compliance (Cdyn) changes in response to aerosol administration of serial dilutions of adenosine or histamine (0.17mg/ml – 20mg/ml). Briefly, rabbits were anesthetized and relaxed with 1.5 ml of a mixture of ketamine

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hydrochloride (35mg/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 Verterinary Supplies, NC) as previously described (Zavala D and Rhodes M, 1973). A polythelene catheter of OD ~2.4mm (Becton Dickinson, 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 endothracheal 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 (Rt) and dynamic compliance (Cdyn) were calculated at isovolumetric and zero flow points. Recording of flow, volume, and pressure were made amplified on an 8-channel Gould 2000W high-frequency amplifier. Cdyn was calculated using total volume and the difference in Ptp at zero flow. Rt was calculated as the ratio of Ptp 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

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computer, as described earlier (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 rabbit was 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 μm in size. Histamine or adenosine aerosol was administered in increasing concentrations (0.17 – 10 mg/ml) with measurements of pulmonary function following for 3 min after each dose. Animals were not exposed to higher doses of histamine or adenosine after their PC30 (concentration (mg/ml) required to produce 30% reduction in Cdyn) 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 Cdyn value with 3 min of treatment. This point was typically achieved within the first 2 min following 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 PC30 to histamine or adenosine.

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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 (early allergic response; EAR) and airway inflammation (late allergic response; 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 anaesthetized 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 (Cdyn) was then measured at 15-min intervals during the next 6 hours 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 one hour before allergen challenge in the same animals following at least a two week washout period. Anesthesia was maintained with a mixture of ketamine/acepromazine (80:20) at a dose of 0.15mg/kg given every 45 min – 1 hour.

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 two weeks (n=5) were aerosolized with histamine as described above to determine their baseline PC30 to histamine and BHR to histamine. Twenty-four hours later, the rabbits were given an aerosol challenge of 2500 AU of house dust mite allergen (HDM). Then BHR measurement with histamine aerosolized challenge was repeated at 24 hr

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following 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 two weeks (n=5) were aerosolized with adenosine as described above to determine their baseline PC30 to adenosine. The following day, one hour 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 hr 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, 15min, 30min, 1hr, 2 hrs, 3hrs, 6hr, 8hrs, and 24hrs following 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 LC/MS/MS 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

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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 hr, 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 minute recovery period prior to challenge with the increasing concentrations of the nonselective AR agonist, 2-chloroadenosine (2-CADO) (5×10^{-5} M 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.

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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 (BioWhittaker Inc (Walkersville MD) expressing the human A_1 AR and membranes from HEK-293 cells expressing the recombinant human A_{2A} and human A_{2B} ARs (Receptor Biology, Inc., Beltsville MD).

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

Human PAECs were obtained from BioWhittaker Inc. (Walkersville, MD) 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 (Clonetics) which contains 2.0% fetal bovine serum. The cells were washed 3 times with PBS and then suspended in lysis buffer (10 mM Tris HCl-pH 7.4 containing 5 mM EDTA, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml benzamidine, 2 µg/ml pepstatin). The cells were homogenized by sonication. The homogenate was centrifuged at 1000 x g at 4°C for 10 minutes. The supernatant was centrifuged at 30000 x g for 45 minutes. The pellet was reconstituted in reconstitution buffer (50 mM Tris HCl pH 7.4, 5 mM EDTA, 10 mM $MgCl_2$, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml benzamidine, 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.

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Determination of the Affinity of L-97-1 for Human A_1 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 HEK 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 which are presented in the following table. Following incubation, the samples were filtered rapidly under vacuum through polyethyleneimine-treated filters and washed 4 times with 3 ml ice-cold buffer using a cell harvester (Skatron). The filters were dried and counted for radioactivity with the use of a liquid scintillation counter (Packard). Two- four experiments were performed and assayed in duplicate.

The affinity of L-97-1 for the recombinant human A_3 AR was determined in another laboratory (Dr. Gary L. Stiles, Duke University Medical Center, Durham NC) in CHO cells expressing the recombinant human A_3 AR with the use of N^6-(4-amino-3-[125I]iodobenzyl)adenosine-5'-*N*-methylcarboxamide, [125I]-AB-MECA as the competing radioligand in competition radioligand binding assays (n = 2).

To further validate the selectivity of L-97-1 for the human A_1 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, alpha 1 and alpha 2,

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peripheral benzodiazepine, non-selective dopamine, glutamate (AMPA, kainate, NMDA agonist, and glycine NMDA sites), strychnine-sensitive glycine, H_1 histamine, H_2 histamine, and H_3 histamine, non-selective central muscarinic, non-selective peripheral muscarinic, non-selective serotonin, and non-selective 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 in order to validate the experiment. Radioactivity was determined with a scintillation counter (Topcount, Packard).

Chemicals

[^{3}H] DPCPX and [^{3}H] CGS 21680 were purchased from NEN Life Science Products, Inc (Boston MA). R-PIA, CCPA, NECA, DPCPX, and all other common reagents of analytical grade were purchased from Sigma Chemical Company (St. Louis, MO). L-97-1 was custom synthesized by ChemSyn Laboratories (Lenexa KS) and provided by Constance N. Wilson, M.D., Chief Scientific Officer, 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 cm^2 is digitized by computer assisted plenometry for each rabbit during a 6 hour period following

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adenosine and allergen challenge and at 24 hours following adenosine, histamine and allergen challenge. The early and late phase responses are determined at 0-1 hours and 1-6 hours, respectively, as previously established in this allergic rabbit model. The percentage change in dynamic compliance (Cdyn) is calculated for each time point for allergen challenge (every 15 minutes for 6 hours). 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 hyperresponsiveness for adenosine and histamine is calculated by determining the concentration of adenosine or histamine (mg/ml) required to reduce the Cdyn by 30% from baseline (PC_{30}). Significance for histamine responses is determined by comparing these values using analysis of variance (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 ± SEM. A value of $p<0.05$ is considered significant.

Radioligand binding data was analyzed by nonlinear regression using a sigmoidal dose-response curve with variable slope (GraphPad Prism version 3.0).

Results from assays for inhibition of human phosphodiesterase enzymes are expressed as a percent of control values and as a percent inhibition of control values obtained in the presence of the test compounds. IC_{50} values (concentration causing a half-maximal inhibition of control values) and Hill coefficients (nH) were determined for

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the reference compounds by non-linear regression analysis of their inhibition curves. These parameters were obtained by Hill equation curve fitting. The IC_{50} values obtained for the reference compounds have passed the required inspections. They are within accepted limits of historic averages obtained ± 0.5 log units.

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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 Cdyn, up to six hours compared to the untreated control group (n = 5). The curve is significant at all time points after 30 minutes (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 PC_{30} (histamine) to 4±1 mg/ml 24 hours following allergen challenge from the baseline PC_{30} of 15±4 mg/ml (no allergen, no drug) ($p<0.05$, n=5). Administration of L-97-1 (10 mg/kg, oral), 1 hour before allergen challenge markedly increased the PC_{30} from 4±1 mg/ml (24 hours following allergen challenge with no drug) to 20±8 mg/ml (24 hours following 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. PC_{30} adenosine increased significantly the baseline PC_{30} from 4.14±0.83 mg/ml 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 hour before adenosine challenge). The PC_{30} after 6 hours (11.67±4.41 mg/ml) and 24 hours (5.83±2.2 mg/ml) of single administration of L-97-1 (10 mg/kg, oral) was

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higher compared to baseline reaching statistical significance at 6 hours compared to the baseline ($p<0.05$, n=5).

Plasma Levels of L-97-1

Table 1 shows the levels of L-97-1 in a 24-hour period following 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 hours following 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 hours following allergen challenge. This lasting effect of L-97-1 at 24 hours 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}M and 10^{-6}M) selectively blocks the contractile responses of 2-CADO (5×10^{-5} M 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 2. L-97-1 has high affinity (580 nM) and high selectivity for the human A_1 AR with no binding to recombinant human A_{2A} or A_{2B} ARs at high concentration ($\leq$ 100 μM). Moreover no binding was demonstrated for L-97-1 ($\leq$ 1 mM) to the recombinant human A_3 AR (data not shown). There was no binding of

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L-97-1 to rat adrenergic, alpha 1 and alpha 2, peripheral benzodiazepine, non-selective dopamine, glutamate (AMPA, kainate, NMDA agonist, and glycine NMDA sites), strychnine-sensitive glycine, H_1 histamine, H_2 histamine, and H_3 histamine, non-selective central muscarinic, non-selective peripheral muscarinic, non-selective serotonin, and non-selective opiate receptors (data not shown).

Phosphodiesterase Inhibition Assays

The effects of L-97-1 (100 µM) and theophylline (100 µM) on the human phosphodiesterase II, III, IV, and V, enzymes are summarized in Table 3 where the IC_{50} values for the reference compounds are also indicated. As opposed to theophylline (100 µM) which inhibits human PDE enzymes II, III, and IV at 19, 28, and 21% inhibition, respectively, L-97-1 (100 µM) does not inhibit human PDE enzymes II, III, IV or V.

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Discussion

In the present study, a rabbit model of allergic asthma was used to study the effects of a novel A_1AR antagonist, L-97-1, on HDM-induced early and late phase allergic responses (EAR and LAR, respectively) 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 (Cdyn) following 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)) which 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 A_1 AR. Moreover, in vitro pharmacology studies in small airways of allergic rabbits suggest that L-97-1 selectively produces its anti-asthma 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 A_1 ARs on ASM are a direct target for adenosine in humans and allergic rabbits (Ali et al., 1994 b; Nyce and Metzger, 1997; Mundell et al., 2001). Moreover, in the allergic rabbit model of asthma, administration of a respiratory antisense

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oligodeoxynucleotide specific for the A_1 AR, EPI 2010, reduced the density of A_1 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 minutes and 6 hours after oral administration of L-97-1. In earlier studies, it was reported that BHR to adenosine in allergic rabbit model of asthma is mainly due to upregulation of A_1 ARs on ASM (Ali et al., 1994 b; 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 A_1 AR antagonist on A_1 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 anti-asthma drugs which are in current use for treatment of human asthma (Ali et al., 1992 a; Ali et al., 1992 b; Ali et al., 1994 c). Both theophylline and beclomethasone administered as inhalational treatments inhibit EAR and LAR responses in this rabbit model of allergic asthma (Ali et al., 1992 b; Ali et al, 1994 c). 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 dependency (Gascoigne et al., 2003; Gozzard et al., 1997; Herd and Page, 1996; Larsen et al., 1984; Metzger et al., 1989). Moreover, adenosine levels are increased in the bronchoalveolar fluid of both humans and rabbits with allergic asthma (Ali et al., 1992 c; Driver et al., 1993).

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We therefore investigated the effect of L-97-1, an A_1 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 hours following allergen challenge, in the allergic rabbit model of asthma. L-97-1 (10 mg/kg) administered one hour before HDM allergen administration significantly increased Cdyn at all time points after 30 minutes up to six hours, thus preventing the decline in Cdyn and blocking both EAR and LAR responses following allergen challenge in rabbits with allergic asthma. The increase in Cdyn during the EAR response can be explained by the direct blocking of A_1 ARs on airway smooth muscle as some of the previous studies have suggested (Ali et al., 1994 b; Nyce and Metzger, 1997). Previously, it was reported that EPI 2010 reduces EAR response in allergic rabbits and decreases the expression of A_1 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 which is lower than that which would produce plasma levels to inhibit PDE enzymes (Ali et al., 1992 b). The inhibitory activity of L-97-1 on A_1 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. Taken together, the results of these studies suggest that L-97-1 may block allergen-induced EAR responses by blocking activation of A_1 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. A_1 ARs may be

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upregulated 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 bamiphylline preferentially produce their anti-asthma effects in humans at concentrations that do not inhibit phosphodiesterase enzymes (Catena et al., 1988 ; Clarke et al., 1989; Foutillan et al., 1983 ;Ginesu et al., 1987 ; 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 bamiphylline produces its anti-asthma effects by selectively blocking A_1 ARs. Bamiphylline binds to the human A_1 AR and human A_{2A} AR with higher affinity for the human A_1 AR (1.93 μM) than the human A_{2a} AR (12.9 μM) and does not bind to human A_{2B} or human A_3 ARs ((≤ 100 μM), personal communication, Constance N. Wilson)). The therapeutic plasma concentration of bamiphylline 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; personal communication, Constance N. Wilson). Moreover, in humans

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theophylline produces its anti-asthma 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 -100 times below that needed to inhibit human PDE enzymes in vitro (Clarke et al., 1989). Compared to bamiphylline and theophylline, L-97-1 binds selectively to the human A_1 AR. Moreover, L-97-1 produces its anti-asthma effects in allergic rabbits at concentrations that are 1000 – 10,000 times less than that needed to inhibit human PDE enzymes.

Taken together these data suggest that L-97-1 is a potent, selective antagonist of A_1 ARs and its efficacy in inhibiting the allergen-induced EAR, LAR responses and attenuating BHR to histamine following allergen challenge and adenosine-induced BHR in allergic asthmatic rabbits is most likely related to its antagonism of A_1 ARs on ASM and perhaps on other cell types, such as inflammatory cell types that play an important role in the LAR response. Activation of A_1 ARs on human neutrophils and macrophages has been shown to produce pro-inflammatory effects (Cronstein et al., 1990; Cronstein et al., 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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Footnotes

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Legends for Figures:

Fig.1 Lung dynamic compliance (Cdyn) in house dust mite (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 hour before HDM administration. Ventilation was monitored in the anesthetized rabbits up to 6 hours 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 is expressed as Mean ± SEM (n=5).

Fig.2 Histamine responses (Histamine PC30 (mg/ml) in rabbits aerosolized with house dust mite (HDM) allergen with or without L-97-1 (10 mg/kg) administered by intragastric tube 1 hour before HDM administration. Control measurement PC 30 histamine was taken without HDM and without prior drug treatment. PC30 histamine was measured again 24 hr after HDM or HDM + L-97-1 treatment. Data is expressed as Mean ± SEM (n=5). *$p<0.05$ as compared to Control; **$p<0.05$ as compared to 24 hrs after HDM treatment (ANOVA).

Fig.3 Adenosine responses (Adenosine PC 30 mg/ml) in allergic rabbits with or without L-97-1. Adenosine PC30 measurements were taken 15 min, 6 hr and 24 hr after administration of L-97-1 (10 mg/kg) by intragastric tube. Control measurements to adenosine in allergic rabbits were taken without house dust mite (HDM) allergen

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challenge and 24 hr before L-97-1 administration. Data is expressed as Mean±SEM (n=5). *p<0.05 as compared to 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 X 10^{-5} M and 10^{-4} M) and histamine (5 X 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 8. Data is expressed as Mean±SEM. *p<0.006 as compared to agonist without L-97-1 (Student's t-test for paired data).

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Assay Table for determination of the Affinity of L-97-1 for Human A_1 and Recombinant Human A_{2A}, and A_{2B} Adenosine Receptors in Inhibition, Competition Radioligand Binding Assays

	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/21.2µg
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/Conc	[3H] DPCPX*/48 nM	[3H] CGS 21680/21 nM	[3H] DPCPX/34 nM
NS Binding/Conc	R-PIA (100 µM)	NECA (50 µM)	NECA (100 µM)
Incubation Time	2 hours	90 minutes	60 minutes
Filter type	GF/C	934/AH	GF/C

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*Concentration of [3H] DPCPX used in A_1 AR radioligand competition binding assay was determined from saturation radioligand binding assays in membranes from human PACES to determine the Kd [(48 nM); average of 2 experiments performed in duplicate)]**;** Adenosine deaminase (AD); [3H] 1,3 dipropyl-8-cyclopentylxanthine (DPCPX); [3H] 2-p-(2-carboxyethyl) phenethylamino-5'-*N*-ethyl-carboxamidoadenosine (CGS 21680), a selective radioligand for the A_{2A} AR; N^6- R-phenylisopropyladenosine (R-PIA); 5'-(N-ethylcarboxamido)-adenosine (NECA) were used to determine nonspecific binding for the A_1 AR, and for the A_{2A}ARs and A_{2B} ARs, respectively.

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Assay Table for Inhibition of Human Phosphodiesterase Enzymes

The assays were performed using the following general procedures:

Assay	Origin	Reference compound
Phosphodiesterase II *(h)*	differentiated U-937 cells	EHNA
Phosphodiesterase III *(h)*	human platelets	milrinone
Phosphodiesterase IV *(h)*	U-937 cells	rolipram
Phosphodiesterase V *(h)*	human platelets	dipyridamole

The experimental conditions are summarized below:

Assay	Substrate	Incubation	Reaction product	Method of detection
Phosphodiesterase II *(h)*	[^{3}H]cAMP (1 μM)	30 min./30°C	[^{3}H]5'AMP	liquid scintillation
Phosphodiesterase III *(h)*	[^{3}H]cAMP (0.1 μM)	30 min./30°C	[^{3}H]5'AMP	liquid scintillation
Phosphodiesterase IV *(h)*	[^{3}H]cAMP (1 μM)	30 min./30°C	[^{3}H]5'AMP	liquid scintillation
Phosphodiesterase V *(h)*	[^{3}H]cGMP (1 μM)	30 min./30°C	[^{3}H]5'GMP	liquid scintillation

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Table 1: Plasma levels (ng/ml) of L-97-1 in a 24-hour period following oral administration of L-97-1 (10mg/kg)

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
SEM	73.04	58.42	37.60	9.40	6.45	4.79	3.81	0.43

Data is presented as Mean±SEM, n = 5

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Table 2 Affinities of L-97-1 for Human A_1 and Recombinant Human A_{2A}, and A_{2B} Adenosine Receptors

Ligand	Human A_1 (^{3}H-DPCPX)			Human A_{2A} (^{3}H-CGS21680)			Human A_{2B} (^{3}H-DPCPX)		
	IC_{50} (μM)	K_i (μM)	N	IC_{50} (μM)	K_i (μM)	N	IC_{50} (μM)	K_i (μM)	N
L-97-1	1.42 ± 0.57	0.58 ± 0.33	4	> 100	ND	3	> 100	ND	3
DPCPX	0.08 ± 0.04	0.04 ± 0.02	4	ND	ND		0.22 + 0.07	0.12 + 0.04	3
CCPA	0.03 ± 0.02	0.02 ± 0.01	2	ND	ND		ND	ND	3
CGS-21680	ND	ND		0.32 ± 0.22	0.16 ± 0.11	3	ND	ND	

ND – Not determined

The following compounds are used as competing radioligands for the A_1 and A_{2A}, and A_{2B} ARs: [3H] 1,3 dipropyl-8-cyclopentylxanthine (DPCPX) (A_1 and A_{2B} ARs) and [3H] 2-p-(2-carboxyethyl) phenethylamino-5'-*N*-ethyl-carboxamidoadenosine (CGS 21680) (A_{2A} ARs). The following compounds are used as reference compounds: DPCPX and 2-chloro-N6-cyclopentyladnenosine (CCPA) are reference compounds for the A_1 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 Ki (measurements of affinity) of the ligand for the AR. The Ki or IC50 are inversely related to the affinity of the ligand for the receptor, *i.e.* the lower the Ki or IC50 the higher the affinity.

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Table 3 Effects of L-97-1 and theophylline on human phosphodiesterase (PDE) enzymes and IC_{50} values for the reference compounds

Assays	L-97-1	Theophylline	Reference compounds		
	100 µM	*100 µM*		*IC_{50} (µM)*	*(nH)*
PDE II *(h)*	-	19	EHNA	4.7	*(0.7)*
PDE III *(h)*	-	28	milrinone	0.39	*(0.8)*
PDE IV *(h)*	-	21	rolipram	0.21	*(0.6)*
PDE V *(h)*	-	-	dipyridamole	0.80	*(1.2)*

For the test compounds, the results are expressed as a percent inhibition of control activity (mean values; n = 2). The symbol - indicates an inhibition of less than 10%.

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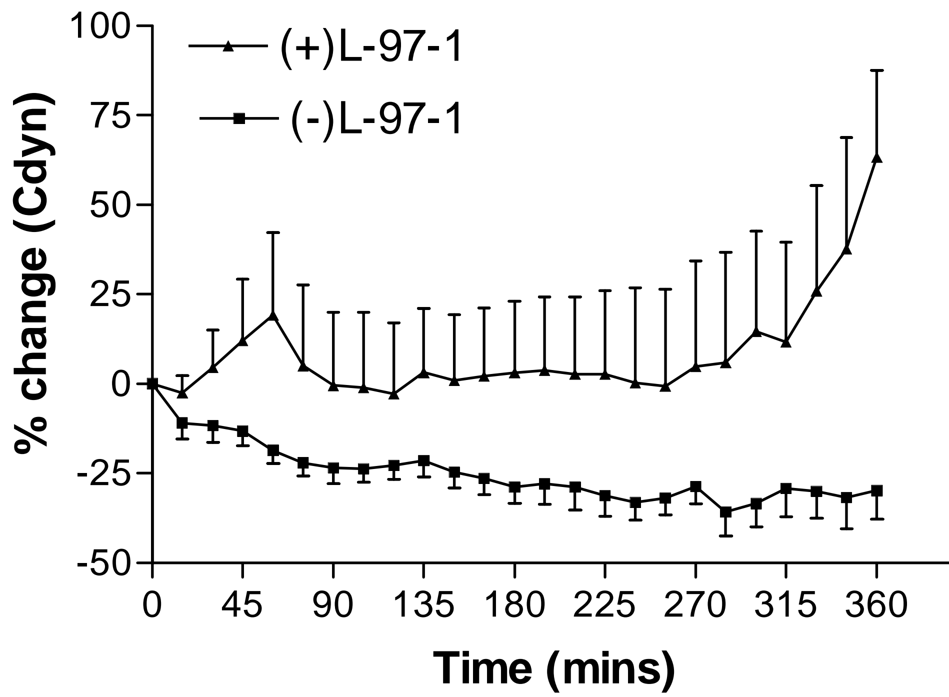

Fig. 1

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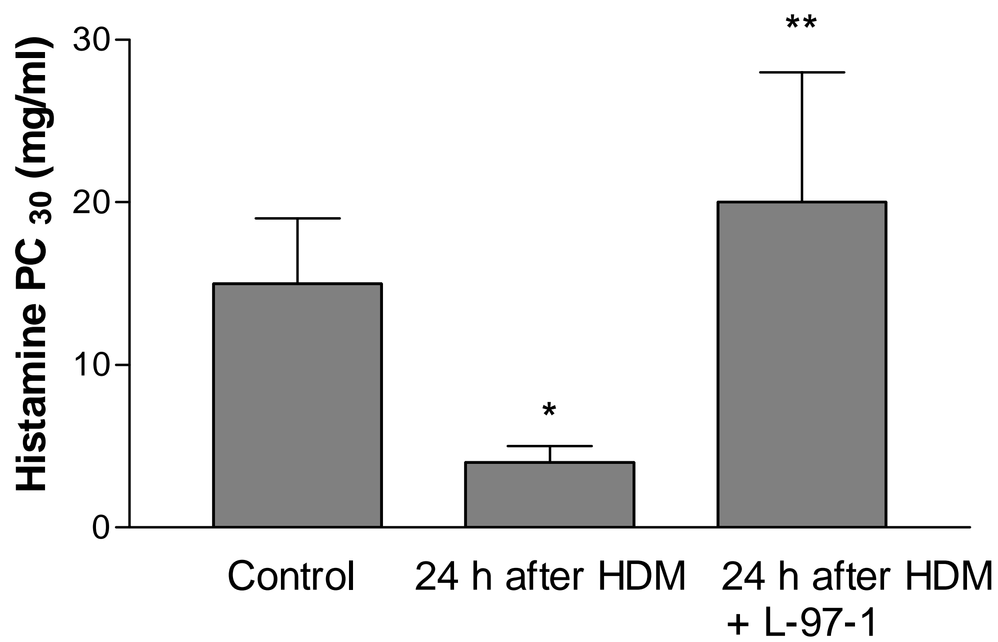

Fig. 2

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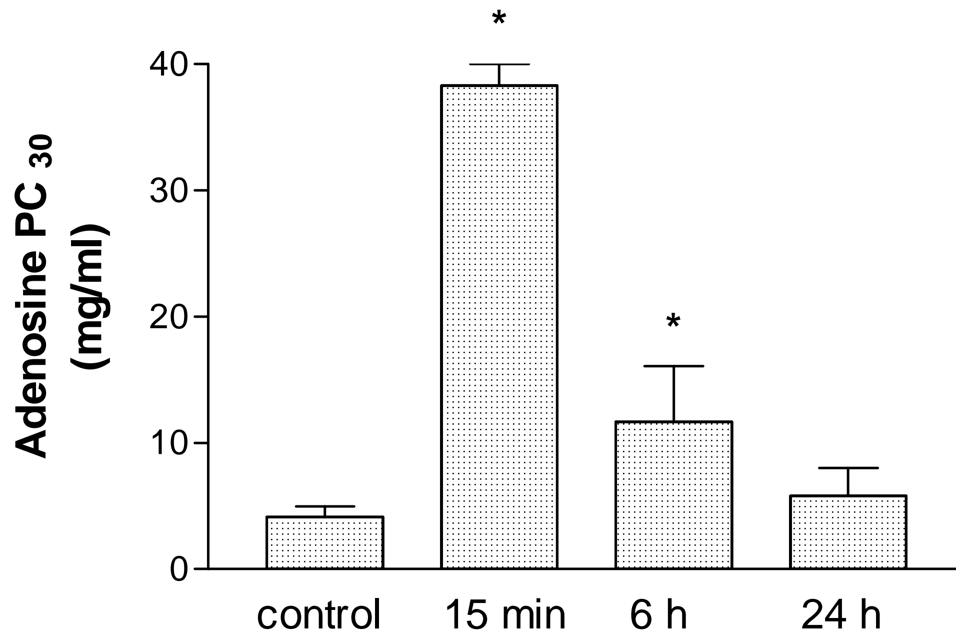

Fig. 3

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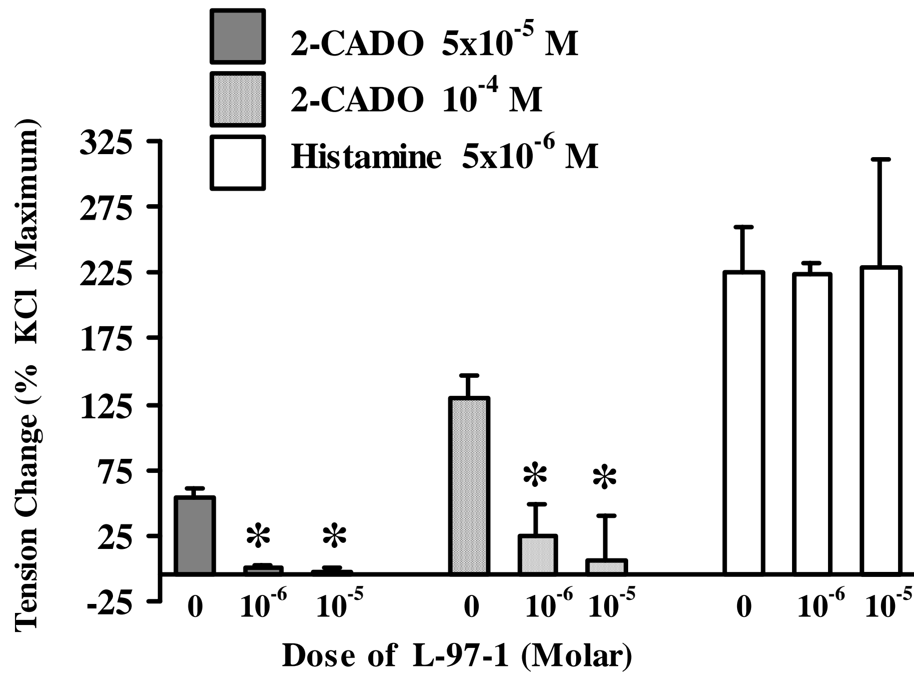

Fig. 4

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