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A small molecule, orally active, $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonist reduces leukocyte infiltration and airway hyper-responsiveness in an experimental model of allergic asthma in Brown Norway rats

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1 $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins are preferentially expressed on eosinophils and mononuclear leukocytes and play critical roles in their recruitment to inflammatory sites. We investigated the effects of TR14035, a small molecule, $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonist, in a rat model of allergic asthma.

2 Actively sensitized rats were challenged with aerosol antigen or saline on day 21, and the responses evaluated 24 and 48-h later. TR14035 (3 mg kg^{-1}, p.o.) was given 1-h before and 4-h after antigen or saline challenge.

3 Airway hyper-responsiveness to intravenous 5-hydroxytryptamine was suppressed in TR14035-treated rats. Eosinophil, mononuclear cell and neutrophil counts, and eosinophil peroxidase and protein content in the bronchoalveolar lavage fluid (BALF) were decreased in TR14035-treated rats. Histological study showed a marked reduction of lung inflammatory lesions by TR14035.

4 At 24-h postchallenge, antigen-induced lung interleukin (IL)-5 mRNA upregulation was suppressed in TR14035-treated rats. By contrast, IL-4 levels in BALF were not significantly affected by TR14035 treatment.

5 IL-4 selectively upregulates vascular cell adhesion molecule-1 (VCAM-1), which is the main endothelial ligand of α_4 integrins. Intravital microscopy within the rat mesenteric microcirculation showed that 24-h exposure to 1 μg per rat of IL-4 induced a significant increase in leukocyte rolling flux, adhesion and emigration. These responses were decreased by 48, 100 and 99%, respectively in animals treated with TR14035.

6 In conclusion, TR14035, by acting on $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, is an orally active inhibitor of airway leukocyte recruitment and hyper-responsiveness in animal models with potential interest for the treatment of asthma.

Introduction

Allergic airway inflammation is characterized by airway and peribronchial leukocyte accumulation that consists of eosinophils and lymphocytes. It is widely accepted that lymphocytes, through the production of Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13, mediate the recruitment and activation of eosinophils (O'Byrne *et al.*, 2004). In allergic airway diseases, eosinophils have been hypothesized to secrete toxic granule proteins and other inflammatory mediators that contribute to the hallmark features of asthma (Kay, 2005).

Leukocyte migration into the lung requires the interaction between surface-adhesion receptors on leukocyte and counter-structures on pulmonary endothelium. The α_4 integrin subunit (CD49d) associates with either the β_1 (CD29) or the β_7 subunit to form the integrin heterodimers very late antigen-4 (VLA-4; $\alpha_4\beta_1$; CD49d/CD29) and $\alpha_4\beta_7$ (Berlin *et al.*, 1993; Carlos & Harlan, 1994). VLA-4 is expressed at significant levels on all circulating leukocytes except neutrophils, which display low level expression but functionally active. VLA-4 binds to vascular cell adhesion molecule-1 (VCAM-1; CD106), a member of the Ig gene superfamily, that is expressed on cytokine-activated endothelial cells, and to the CS-1 sub-domain of human fibronectin. $\alpha_4\beta_7$ is expressed on a subset of T and B cells, natural killer, and eosinophils. It binds to the mucosal vascular adressin (MAdCAM-1), a member of the Ig and mucin-like families of adhesion molecules, as well

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as to VCAM-1 and fibronectin (Berlin *et al.*, 1993; Erle *et al.*, 1994).

One approach to inhibit infiltration of eosinophils into tissues is by interfering with cell adhesion molecules that mediate the rolling and the firm adhesion of these cells prior to their emigration to the extravascular tissue (Lobb *et al.*, 1996; Wardlaw, 1999). In this context, VCAM-1, the main endothelial ligand of α_4 integrins can be upregulated in the airways following antigen challenge (Bentley *et al.*, 1993; Ohkawara *et al.*, 1995). Thus, monoclonal antibodies (mAbs) directed against α_4 integrins have been proved to inhibit not only the migration of eosinophils into the airways but also allergen-induced airway hyper-responsiveness in different animal models of asthma including guinea-pigs (Pretolani *et al.*, 1994; Kraneveld *et al.*, 1997), rats (Rabb *et al.*, 1994; Richards *et al.*, 1996), rabbits (Gascoigne *et al.*, 2003), sheep (Abraham *et al.*, 1994) and mice (Henderson *et al.*, 1997; Kanehiro *et al.*, 2000, 2001; Borchers *et al.*, 2001a, b). More recently, different companies have developed small molecules that antagonize α_4 integrin function and demonstrated that these compounds can also inhibit leukocyte infiltration and airway hyper-responsiveness in different *in vivo* animal models of pulmonary inflammation (Abraham *et al.*, 1997; 2000; Kudlacz *et al.*, 2002; Huryn *et al.*, 2004). Furthermore, some of these compounds are currently undergoing clinical trials for the treatment of asthma (Vanderslice *et al.*, 2004).

In the present study, we have evaluated the effect of a synthetic small molecule, that is a dual $\alpha_4\beta_1$ and $\alpha_4\beta_7$ antagonist, TR14035 (Sircar *et al.*, 2002), in a rat model of allergic asthma. This compound was proved to be effective blocking $\alpha_4\beta_7$-dependent adhesion of lymphocytes to high endothelial venules (Egger *et al.*, 2002), and was reported to have reached phase I clinical trial for asthma (Vanderslice *et al.*, 2004). In addition, since IL-4 causes VCAM-1 upregulation without affecting the expression of intercellular adhesion molecule-1 (ICAM-1) or E-selectin, we have employed intravital microscopy to test the effect of TR14035 on IL-4-induced leukocyte–endothelial cell interactions in the mesenteric microvasculature of the rat.

Methods

Animal model and experimental groups

Male Brown Norway rats (250–300 g) were supplied by B&K Universal (Barcelona, Spain) and kept at room temperature of 22°C under a 12 h phase light–dark cycle and fed on A04 pellets (Panlab, Barcelona, Spain). Drinking water was freely available. The experimental protocols were approved by the institutional ethics committee and comply with Spanish and European community regulations for use of laboratory animals.

Animals were actively sensitized as previously outlined (Blesa *et al.*, 2003). Sensitized animals were randomly distributed into negative control (vehicle + saline; NS), positive control (vehicle + antigen; SZ) and drug-treated (NS + TR14035, SZ + TR14035) groups. TR14035 (3 mg kg^{-1}) was administered orally by gavage at 1 h before and 4 h after challenge with antigen or saline. In an additional group of experiments, TR14035 was orally administered at 10 mg kg^{-1} with the same schedule. TR14035 was solved in NaOH 0.5 N and diluted as appropriate in phosphate-buffer saline. The oral

route was selected as convenient in the clinical setting. The dose level and schedule were based on pilot studies in this laboratory.

Animals were anesthetized and instrumented for measurement of lung resistance (R_L) and dynamic compliance (C_{dyn}) as previously outlined (Suchankova *et al.*, 2005). Unanesthetized rats were exposed to ovalbumin (1% in saline) or saline aerosol for 15 min. The time course of airway hyper-responsiveness in antigen exposed Brown Norway rats has been previously examined (Elwood *et al.*, 1992) and the response at 24 h was selected on this basis. 5-Hydroxytryptamine (5-HT) was used to assess airways hyper-responsiveness since it produces a direct and reproducible bronchoconstrictor response in rats (Pauwels *et al.*, 1985). Thus, airway responsiveness was determined in anesthetized animals from dose–response curves to 5-HT (5–50 μg kg^{-1}, i.v.). After measurement of airway responsiveness (24 h post-challenge) or in additional experiments at 48 h after challenge with antigen, animals were killed with an overdose of sodium thiopental. Then, bronchoalveolar cells were collected in two successive lavages using 6 ml aliquots of sterile saline at room temperature instilled and recovered through a polyethylene tracheal cannula. Cell suspensions were concentrated by low-speed centrifugation, and the cell pellet resuspended. Total cell counts were made in a haemocytometer. Differential cell counts were determined from cytospin preparations by counting 300 cells stained with May–Grünwald–Giemsa. Because the yield of the instilled fluid was equivalent in all experimental groups ($\geqslant$85%), the results are expressed as absolute cell counts per ml of lavage fluid. The cell-free supernatant was used for biochemical determinations. The levels of free eosinophil peroxidase in the supernatant from bronchoalveolar lavage fluid (BALF) was determined as a marker of eosinophil activation, according to a previously described method (Strath *et al.*, 1985) with modifications (Pons *et al.*, 2000), and the results expressed as the optical density measured at 490 nm in a Microplate Autoreader (EL309, Bio-Tek Instruments). Total protein concentration was measured by a standard technique (BCA protein assay reagent kit; Pierce, Rockford, U.S.A.). IL-4 was measured by enzyme-immunoassay kit (Diaclone Research, Besançon, France).

For histological studies, lung was perfused and tissue blocks placed in formalin, cut into 4-μm-thick serial sections, and stained with haematoxylin–eosin or with toluidine blue to identify inflammatory cells as previously outlined (Serrano-Mollar *et al.*, 2003).

The IL-5 mRNA transcripts were measured by real-time quantitative reverse transcriptase–polymerase chain reaction (RT–PCR). The method used for obtaining quantitative data of relative gene expression was the comparative C_t method ($\Delta\Delta C_t$ method) as described by the manufacturer (PE-ABI PRISM 7700 Sequence Detection System; Perkin-Elmer Applied Biosystems; Foster City, CA, U.S.A.) and previously reported (Sanz *et al.*, 2005). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as the endogenous control gene. Lung tissue samples were obtained at 24 h postchallenge as an appropriate time point to measure antigen-induced IL-5 mRNA upregulation in this model (Huang *et al.*, 2001; Underwood *et al.*, 2002). IL-5 mRNA was measured since no ELISA kit for rat IL-5 is available, assuming that increases in IL-5 mRNA expression will translate into changes in protein (Underwood *et al.*, 2002). Total RNA was extracted

from lung tissue homogenates by using TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN, U.S.A.). Reverse transcription of RNA to generate cDNA was carried out by using TaqMan Reverse Transcription Reagents (Applied Biosystems) and PCR was performed by using TaqMan Universal PCR Mix (Applied Biosystems) as indicated by the manufacturer. TaqMan primer-probe sets for the following gene were obtained from Applied Biosystems (Taqman Assay-on-Demand™ Gene Expression Products): IL-5 (Rn01459975_m1). The PCR primer for rat GAPDH was designed using the Primer Express software (PE Biosystems, Morrisville, NC, U.S.A.) according to the published rat GAPDH cDNA sequence (GenBank NM17008) as previously reported (Blesa *et al.*, 2003).

Intravital microscopy

Male Brown Norway rats (250–300 g) were fasted for 20–24 h prior to experiments with free access to water. The animals were anesthetized with sodium pentobarbital (65 mg kg⁻¹, i.p.). A tracheotomy was performed to facilitate breathing and the right jugular vein was cannulated for intravenous administration of additional anesthetic as required. The right carotid artery was cannulated to monitor systemic arterial blood pressure through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA, U.S.A.).

A midline abdominal incision was made and a segment of the mid-jejunal mesentery exteriorized and carefully placed on an optically clear viewing pedestal to allow transillumination of a 3 cm² segment of the mesenteric microvasculature. The temperature of the pedestal was maintained at 37°C. The exposed intestine was continuously superfused with a bicarbonate buffer saline (BBS, pH 7.4, 2 ml min⁻¹, 37°C) and covered with a BBS-soaked gauze to prevent evaporation. Mesenteric microcirculation was observed through an orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Netherlands) with a ×20 objective lens (Nikon SLDW) and a ×10 eyepiece as previously described (Sanz *et al.*, 2002). A video camera (Sony SSC-C350P, Koeln, Germany) mounted on the microscope projected the image onto a color monitor (Sony Trinitron PVM-14N2E) and the images were captured on videotape (Sony SVT-S3000P) with superimposed time and date for subsequent playback analysis. The final magnification of the image on the monitor was ×1300.

Single unbranched mesenteric venules with diameters ranging between 25 and 40 μm were studied. Venular diameter (D_v) was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX, U.S.A.). Centerline red blood cell velocity (V_{rbc}) was also measured on-line with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University). Venular blood flow was calculated from the product of mean red blood cell velocity ($V_{mean} = V_{rbc} \ 1.6^{-1}$) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8 \times (V_{mean}/D_v)\,s^{-1}$, in which D_v is venular diameter (House & Lipowsky, 1987).

The number of rolling, adherent and emigrated leukocytes was determined off-line during playback analysis of videotaped images. Rolling leukocyte flux was determined by counting the number of leukocytes rolling passing a fixed reference point in the microvessel per min. The same reference point was used throughout the experiment as leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. V_{wbc} was determined by measuring the time required for a leukocyte to traverse a distance of 100 μm along the length of the venule and was expressed as μm s⁻¹. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period equal to or exceeding 30 s. Adherent cells were expressed as the number per 100 μm length of venule. Leukocyte emigration was expressed as the number of white blood cells per microscopic field.

Experimental protocol

Animals were first sedated with ether and later i.p. injected with 5 ml of saline or a 5 ml solution of recombinant rat IL-4 (1 μg rat⁻¹, equivalent to ∼5000 U of IL-4/animal). In a previous study, this dose of IL-4 caused significant eosinophil accumulation in the rat skin (Sanz *et al.*, 1998). In each experimental group, 24 h after the i.p injection of the agent under investigation, measurements of leukocyte rolling flux, velocity, adhesion, emigration, mean arterial blood pressure (MABP), V_{rbc}, shear rate and diameter were obtained and recorded for 5 min. In another set of experiments, TR14035 (3 mg kg⁻¹) was administered orally by gavage at 1 h before and 4 h after saline or IL-4 i.p. injection and responses were evaluated 24 h after the administration of the stimuli. To determine the effect of TR14035 on levels of circulating leukocytes, blood samples were taken from the rats once the experiment using intravital microscopy was completed.

Statistical analysis

Data are presented as mean ± s.e.m. of *n* experiments. Statistical analysis of data was carried out by analysis of variance (ANOVA) followed by Bonferroni test (GraphPad Software Inc, San Diego, CA, U.S.A.). Significance was accepted when $P < 0.05$.

Materials

TR14035 (*N*-(2,6)-dichlorobenzoyl)-(L)-4-(2′6′-bis-methoxy-phenyl)phenylalanine) was synthesized by Uriach (Barcelona, Spain) as previously outlined (Sircar *et al.*, 2002). Recombinant rat IL-4 was from PeproTech, London, U.K. Other drugs were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Results

Effect of TR14035 on antigen-induced airway hyper-responsiveness and inflammation

TR14035 was tested in an experimental model of allergic asthma in Brown Norway rats. At 24 h post-antigen challenge, untreated sensitized rats exhibited augmented airway responses to 5-HT compared with untreated saline-challenged animals (Figure 1). No significant differences in airway responses to 5-HT were found between untreated and TR14035-treated animals challenged with saline. By contrast,

administration of TR14035 to antigen-challenged rats supressed airway hyperer-reponsiveness to 5-HT (Figure 1).

To asses the effects of TR14035 on the development of allergic inflammation after ovalbumin challenge, inflammatory cells in BALF were measured. At 24 h after challenge,
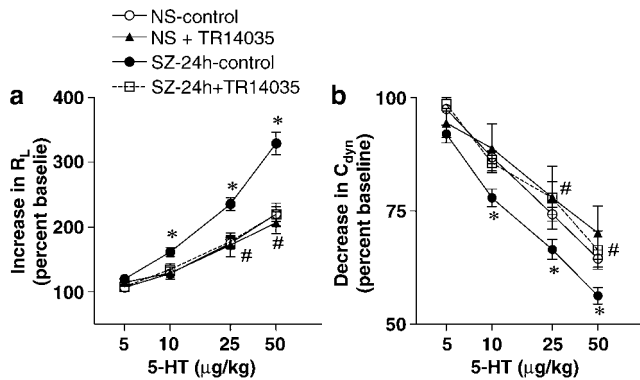

Figure 1 The oral treatment with TR14035 ($3 \, \text{mg} \, \text{kg}^{-1}$, 1 h before and 3 h after antigen challenge), an $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonist, produced a significant decrease of the airways hyper-responsiveness to 5-hydroxytryptamine (5-HT) in an experimental model of allergic asthma in Brown Norway rats. The airway responses to intravenous 5-HT are expressed as increases in lung resistance (R_L) (a) and decreases in lung compliance (C_{dyn}) (b) obtained 24 h after challenge with antigen or saline. The experimental groups are: saline challenge without drug treatment (i.e. negative control; NS), antigen challenge without drug treatment (i.e. positive control; SZ), saline challenge in TR14035-treated rats (NS + TR14035), and antigen challenge in TR14035-treated rats (SZ + TR14035). Points are mean ± s.e.m. of seven animals in each group except studies at 48 h which were carried out in five animals. $*P < 0.05$ NS vs SZ, $^\#P < 0.05$ SZ vs SZ + TR14035.

significant increases in the number of total cells as well as eosinophils, neutrophils, lymphocytes and macrophages were found in the BALF of untreated animals exposed to antigen compared with the counts in saline-challenged rats (Figure 2). Again, no differences in the number of these cells were found in the BALF of untreated and TR14035-treated saline-challenged animals. When the dual $\alpha_4\beta_1/\alpha_4\beta_7$ antagonist was administered to allergen-exposed rats, eosinophil, neutrophil, lymphocyte and macrophage infiltration in the BALF was reduced by 64, 66, 88 and 95% respectively, compared with the infiltration found in the untreated group. Additional experiments at 48 h postantigen challenge also demonstrated the ability of TR14035 to decrease cell counts in BALF (Figure 2).

Light microscopic evaluation of lung tissues collected from saline-challenged rats either untreated or treated with TR14035 showed occasional mild inflammatory changes with very few eosinophils. Lungs from sensitized rats challenged with antigen and examined 24 and 48 h later showed multiple areas of inflammation in which blood vessels and bronchioles were surrounded by eosinophils, scattered lymphocytes and some macrophages, neutrophils and mast cells. Pulmonary lesions in antigen-challenged sensitized rats treated with TR14035 showed a marked decrease in the number and intensity of the clusters of leukocytic inflammatory lesions (Figure 3).

The antigen-induced augmentation of EPO activity in BALF measured at 24 h postovalbumin challenge is considered a marker of eosinophil activation. Consistent with the results encountered for eosinophils in BALF, the augmented EPO in untreated antigen-challenged animals was also significantly reduced in TR14035-treated rats by 69% (Figure 4).
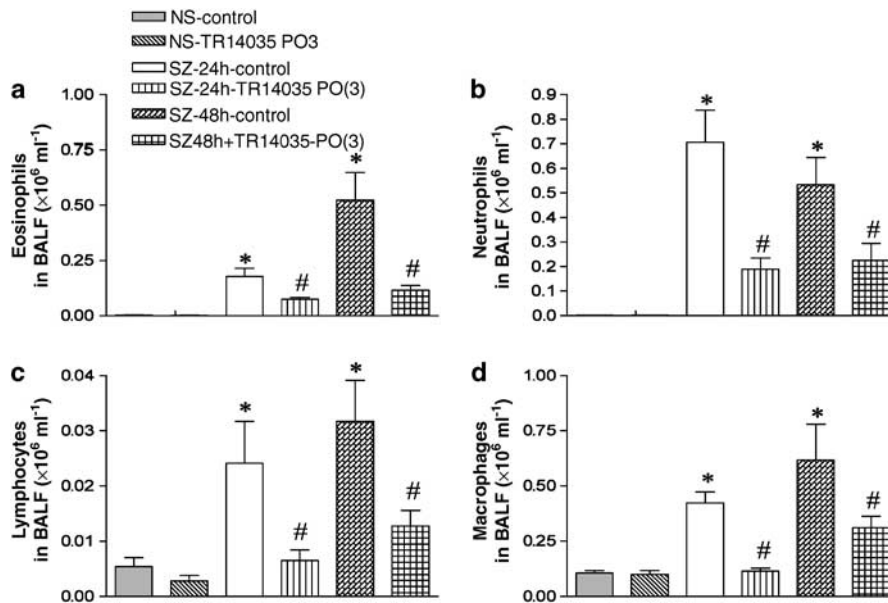

Figure 2 The oral treatment with TR14035 ($3 \, \text{mg} \, \text{kg}^{-1}$, 1 h before and 3 h after antigen challenge), an $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonist, produced a significant decrease of the cell counts for eosinophils (a), neutrophils (b), lymphocytes (c) and macrophages (d) in bronchoalveolar lavage fluid (BALF) in an experimental model of allergic asthma in Brown Norway rats. The cell counts were obtained 24 and 48 h after challenge with antigen or saline in the experimental groups outlined in Figure 1. Total cell counts were 0.11 ± 0.01, 0.11 ± 0.02, $1.28 \pm 0.18^*$, $1.71 \pm 0.40^*$, $0.42 \pm 0.10^\#$ and $0.67 \pm 0.13^\# \times 10^6 \, \text{cells} \, \text{ml}^{-1}$ for NS, NS + TR14035, SZ-24 h, SZ-48 h, SZ-24 h + TR14035, and SZ-48 h + TR14035, respectively. Data are mean ± s.e.m. of seven animals in each group. $*P < 0.05$ vs NS; $^\#P < 0.05$ vs the corresponding SZ group.
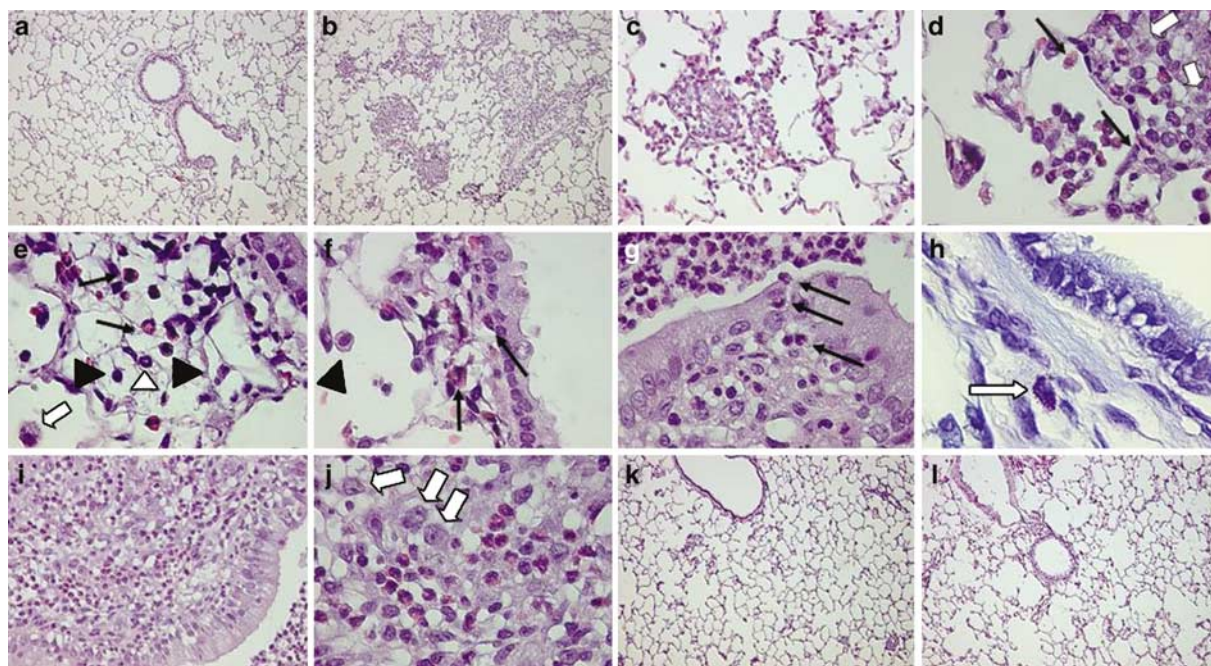
Figure 3 Representative photomicrographs of lung histology in nonsensitized animals (a), sensitized animals at 24 h (b–h) and 48 h (i, j) postantigen challenge, and sensitized animals orally treated with the $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonist TR14305 (3 mg kg^{-1} given 1 h before and 3 h after antigen challenge) at 24 h (k) and 48 h (l) after challenge with antigen in an experimental model of allergic asthma in Brown Norway rats. All lung sections were stained with haematoxylin–eosin except panel h that shows a toluidine blue staining. At 24 h after antigen challenge, a marked peribronchial and perivascular infiltration of inflammatory cells extending into the adjacent pulmonary alveoli was evident in vehicle-treated rats (b–c). Eosinophils (closed arrows) are shown in inflammatory foci (d–f) and in the epithelium (g). Macrophages (open arrows), scattered lymphocytes (closed triangles), neutrophils (open triangle) and mastocytes (indicated by arrow in panel h) are also found in the inflammatory lesions. Lung tissue demonstrates severe inflammatory reactions at 48 h after antigen challenge (i, j). Treatment with TR14035 produced a marked inhibition of inflammatory cell infiltration in the peribronchial and perivascular areas and pulmonary alveoli at 24 and 48 h after antigen challenge (k, l). Magnification of panels × 10 (a, b, k, l), × 40 (c, i) and × 100 (d–h, j).
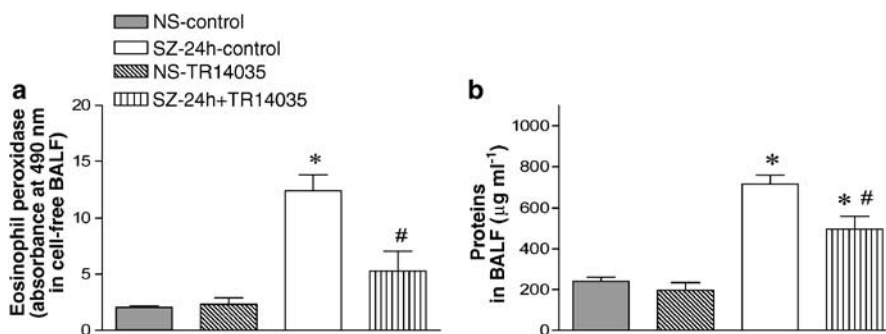

Figure 4 The oral treatment with TR14035 (3 mg kg^{-1}, 1 h before and 3 h after antigen challenge), an $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonist, produced a significant decrease of eosinophil peroxidase activity (a) and proteins (b) in bronchoalveolar lavage fluid (BALF) in an experimental model of allergic asthma in Brown Norway rats. Values were obtained at 24 h after challenge with antigen or saline. Experimental groups are as indicated in Figure 1. Columns are mean ± s.e.m. of seven animals in each group. *$P < 0.05$ vs NS; #$P < 0.05$ vs SZ.

Protein levels in BALF were significantly increased at 24 h post-antigen challenge in the antigen-challenged group, and treatment with TR14035 decreased this response by 46% (Figure 4). Administration of TR14035 to saline-exposed animals did not modify BALF protein content. These results suggest that the airway microvascular leakage after antigen-challenge in sensitized animals is in part related to the infiltration of inflammatory cells, since blockade of α_4-integrin function reduced this response.

In addition, we determined the IL-5 mRNA in lung tissue and the concentration of IL-4 in BALF. After sensitization and challenge with antigen, an increase in IL-5 mRNA and IL-4 protein was observed. Treatment with TR14035 decreased significantly the upregulated IL-5 mRNA but decrease in IL-4 protein failed to achieve statistical significance (Figure 5). TR14035 failed also to decrease IL-4 protein in BALF collected at 48 h postantigen challenge. In additional studies with sensitized rats treated with a higher dose of TR14035 (10 mg kg^{-1}), no significant decrease of IL-4 protein was found at 24 h postantigen challenge (25.4 ± 6 vs 19.1 ± 3.8 pg ml^{-1} in the absence and presence of TR14035, respectively; $n = 5$ in each group, $P > 0.05$). TR14035 administration had no effect
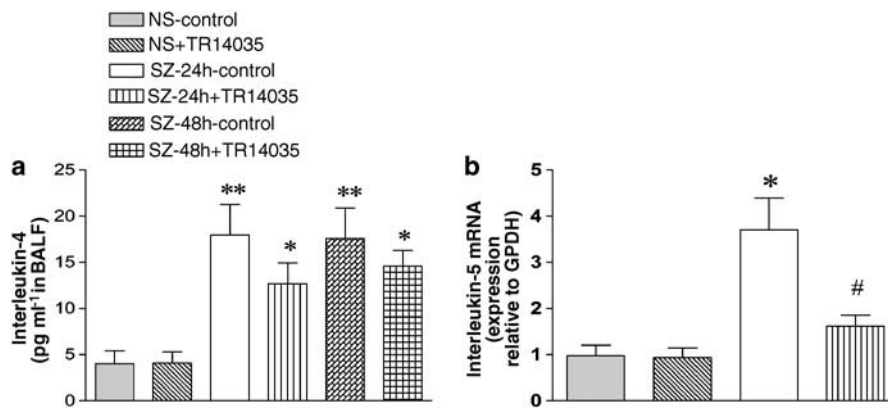
Figure 5 Effect of oral treatment with TR14035 (3 mg kg^{-1}, 1 h before and 3 h after antigen challenge), an $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonist, on antigen-induced increase of interleukin-4 concentration in bronchoalveolar lavage fluid (BALF) (a) and interleukin-5 mRNA transcript in lung tissue (b) in a model of allergic asthma in Brown Norway rats. Determination of interleukin-4 levels in BALF was done by enzyme-immunoassay, and quantification of the mRNA levels of interleukin-5 was measured by real-time quantitative RT–PCR using the comparative C_t method ($\Delta\Delta C_t$ method) and expressed as relative to GAPDH. The C_t values for GAPDH were similar in the different samples, thus confirming the value of this housekeeping gene as endogenous control. Data are shown for the following experimental groups: saline challenge without drug treatment (i.e. negative control; NS), antigen challenge without drug treatment (i.e. positive control; SZ), saline challenge in TR14035-treated rats (NS + TR14035), and antigen challenge in TR14035-treated rats (SZ + TR14035). Measurements are made at 24 and 48 h after challenge with antigen as indicated. Columns are mean ± s.e.m. of seven (NS and SZ at 24 h for IL-4) and five (SZ at 48 h for IL-4 and SZ at 24 h for IL-5) animals in each group. $*P < 0.05$ NS vs SZ, $^{\#}P < 0.05$ SZ vs SZ + TR14035.

on lung IL-5 mRNA or BALF IL-4 protein in saline-challenged animals (Figure 5).

Intravital microscopy in rat mesentery

Since TR14035 did not significantly affect the production of IL-4 in antigen-challenged animals, it is likely that this dual $\alpha_4\beta_1/\alpha_4\beta_7$ antagonist exerts part of its anti-inflammatory activity by inhibiting the interaction of α_4-integrins with VCAM-1. IL-4 causes P-selectin expression through a transcriptionally dependent mechanism with kinetics that are delayed compared with the action of other cytokines such as TNF-α and VCAM-1 upregulation (Yao *et al*., 1996). To investigate such possibility, intravital microscopy was used to examine leukocyte trafficking in the mesentery as leukocyte–endothelial cell interactions would be expected to precede the tissue accumulation of leukocytes. Figure 6 shows the effect of TR14035 on IL-4-induced leukocyte–endothelial cell interactions. After 24 h of i.p. injection of 1 μg per rat of IL-4, significant increases in leukocyte rolling flux, adhesion and emigration, and significant decreases in the V_{wbc} were detected compared to values obtained in the untreated saline group. Treatment with TR14035 decreased IL-4-induced leukocyte rolling flux by 48%, but this effect did not reach statistical significance (Figure 6a). In contrast, it significantly increased the reduction in the V_{wbc} elicited by IL-4 (Figure 6b). Leukocyte adhesion and emigration were abolished by the dual $\alpha_4\beta_1/\alpha_4\beta_7$ antagonist treatment after 24 h exposure to IL-4 (100 and 99% inhibition, respectively; Figure 6c and d). None of these treatments had significant effects on circulating leukocyte counts, MABP and shear rate (Table 1).

Discussion

TR14035 is an *N*-benzoyl-L-biphenylalanine derivative endowed with a potent dual inhibitory activity on $\alpha_4\beta_1$ and $\alpha_4\beta_7$

integrins with IC$_{50}$ values of 87 and 7 nM, respectively (Sircar *et al*., 2002). The potency of this compound for blocking the binding of human and rodent $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins to soluble ligands was similar in both animal species (Egger *et al*., 2002). In the present study, we have evaluated the effects of the oral administration of TR14035 in an animal model of allergic asthma.

Antigen-induced airway inflammation has been studied extensively in animal models. In particular, Brown Norway rats produce high levels of IgE in response to active immunization (Pauwels *et al*., 1979), develop early and late airway responses (Martin *et al*., 1993), and airway hyper-responsiveness and increases of eosinophil, lymphocyte and neutrophil counts in BALF (Elwood *et al*., 1992). In this study, oral administration of TR14035 to sensitized Brown Norway rats suppressed airway hyper-responsiveness to 5-HT and reduced the number of eosinophils, neutrophils, lymphocytes and macrophages in BALF. In addition, protein content and eosinophil peroxidase were diminished. In parallel to these beneficial effects, TR14035 also improved the inflammatory lesions assessed by histological examination of lung sections. However, although IL-4 levels in BALF tended to be reduced by the administration of the dual $\alpha_4\beta_1/\alpha_4\beta_7$ antagonist, differences failed to reach statistical significance.

Neutralizing antibodies against α_4-integrins ameliorate various aspects of late phase allergic airway responses such as inflammatory cell infiltration and hyper-responsiveness (Abraham *et al*., 1994; Foster, 1996; Kanehiro *et al*., 2000; Kudlacz *et al*., 2002). These effects have made $\alpha_4\beta_1$/VCAM-1 interaction an attractive target for the development of orally active, small molecule antagonists (Huryn *et al*., 2004). In this context, several $\alpha_4\beta_1$ antagonists have been effective in inhibiting these responses in animal models of asthma (Abraham *et al*., 1997; 2000; Kudlacz *et al*., 2002). In keeping with these results, our study showed that the oral administration of TR14035 markedly reduced the augmented lung resistance and dynamic compliance responses to intravenous
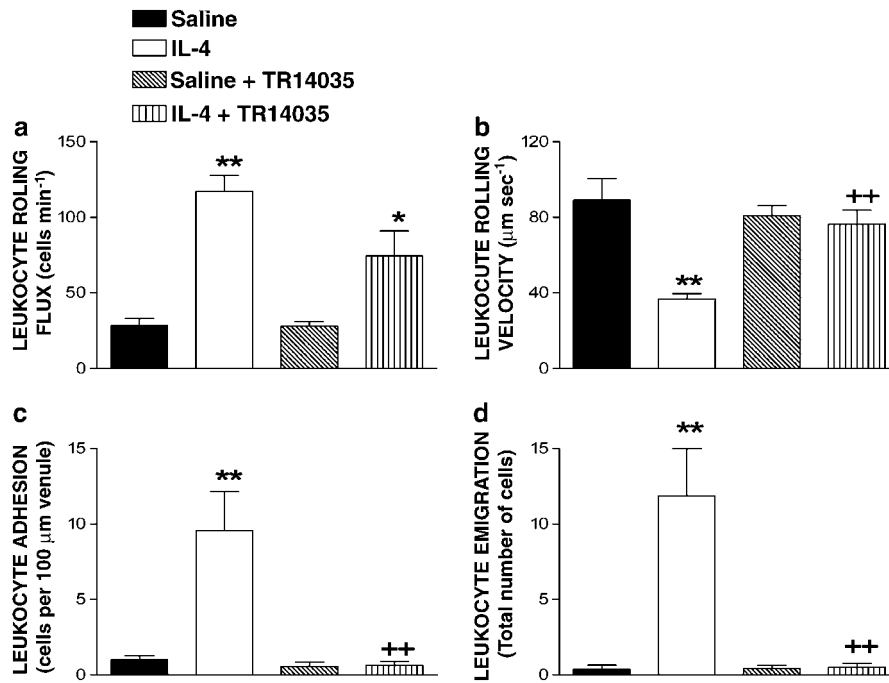
Figure 6 Effect of the oral treatment with TR14035 ($3 \, mg \, kg^{-1}$, 1 h before and 3 h after antigen challenge), an $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonist, on IL-4-induced leukocyte rolling flux (a), leukocyte rolling velocity (b), leukocyte adhesion (c) and leukocyte emigration (d) in Brown Norway rat mesenteric postcapillary venules. Parameters were measured 24 h after i.p. injection of 5 ml of saline or 5 ml of IL-4 (1 μg/rat) in the following experimental groups: untreated rats exposed to saline (negative control); TR14035 treated rats exposed to saline; untreated rats exposed to IL-4 (positive control); and IL-4-exposed rats treated with TR14035. Data are mean ± s.e.m. of 7–8 animals per group; *$P < 0.05$ or **$P < 0.01$ compared to negative control; +$P < 0.05$ or ++$P < 0.01$ compared to positive control.

Table 1 Haemodynamic parameters in vehicle and TR14035-treated animals after 24 h saline and 5 ml of IL-4 (1 μg rat^{-1}) exposure

	Leukocyte counts (cells μl^{-1})	MABP (mmHg)	Shear rate (s^{-1})
Treatment	24 h	24 h	24 h
Saline	3956 ± 719	109.8 ± 3.7	585.2 ± 51.6
TR14035 + saline	2887 ± 743	110.9 ± 5.4	428.4 ± 76.0
IL-4	3998 ± 979	113.1 ± 5.5	455.5 ± 78.0
TR14035 + IL-4	3060 ± 808	107.2 ± 5.6	483.0 ± 64.2

Values are mean ± s.e.m. of 7–8 animals in each group. No significant changes among the different groups were observed.

5-HT, as well as the eosinophil accumulation in BALF at 24 h after allergen challenge. Despite these consistent findings, treatment with an anti-VLA-4 antibody (Henderson *et al.*, 1997) or a VLA-4 antagonist (Koo *et al.*, 2003) prevented eosinophil recruitment but did not suppress allergen-induced airway hyper-responsiveness. Conversely, Rabb *et al.* (1994) reported a decrease in airway hyper-responsiveness without reduced pulmonary leukocyte infiltration after antigen challenged in sensitized rats receiving an anti-VLA-4 antibody.

In the present study, the maximum inhibition of BALF eosinophil infiltration was ~64%. The lack of total inhibition of eosinophil influx by TR14035 is in agreement with previous observations with an α_4 integrins antibody or $\alpha_4\beta_1$ antagonists tested in a murine model of allergen-induced pulmonary inflammation (Kudlacz *et al.*, 2002), and suggests that cell adhesion molecule interactions other than $\alpha_4\beta_1$/VCAM-1

pathway are involved in the eosinophil trafficking from the blood vessel to the extravascular space. In this regard, administration of antibodies against both β_2 and α_4 integrins have been shown to cause a total inhibition of eosinophil infiltration in a rat model of allergic pulmonary inflammation (Schneider *et al.*, 1999). Indeed, ICAM-1, the main endothelial ligand for β_2 integrins, is clearly upregulated at sites of allergic inflammation (Montefort *et al.*, 1992).

Eosinophils also express $\alpha_4\beta_7$ integrin (Erle *et al.*, 1994; Walsh *et al.*, 1996), but its role in allergic asthma has been scarcely studied. The inhibition of BAL eosinophilia by selective antagonist of $\alpha_4\beta_1$ integrin (Kudlacz *et al.*, 2002) is of similar magnitude (~60%) than that produced by $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonists. Therefore, it appears that this cell adhesion molecule do not play an important role in eosinophil infiltration into the airways. However, although VCAM-1 is more effective than MAdCAM-1 in supporting eosinophil rolling, β_1 and β_7 mAbs inhibit eosinophil rolling on VCAM-1 and fibronectin most efficiently in combination (Sriramarao *et al.*, 2000). Furthermore, full inhibition of eosinophil adhesion to fibronectin has been reported to require the combination of β_1 and β_7 mAbs (Matsumoto *et al.*, 1997), and also, *in vitro* studies suggest that $\alpha_4\beta_7$ integrin may be important for eosinophil survival (Meerschaert *et al.*, 1999).

On the other hand, we have observed a significant increase in neutrophil influx in sensitized Brown Norway rats after antigen-challenge. Interestingly, oral treatment with TR14935 markedly reduced (69%) the number of these cells in the BALF 24 h after antigen challenge. Consistent with our observations, in a sheep model of allergic inflammation,

another group found increases in the number of infiltrating neutrophils, which were decreased by the administration of BIO-1211, an $\alpha_4\beta_1$ integrin antagonist (Abraham *et al.*, 2000). These results are not surprising since rat neutrophils express functional α_4- and β_1-integrins (Davenpeck *et al.*, 1998), which would explain this observation.

Another finding was the capability of TR14035 treatment of inhibiting EPO activity in the BALF of antigen-challenged sensitized rats. TR14035 inhibited eosinophil number and EPO to a similar extent (64 and 69%, respectively); therefore, we cannot assure that TR14035 is inhibiting eosinophil activation since the reduction in EPO is likely a consequence of the reduced eosinophil numbers. Nevertheless, an inhibition of eosinophil activation by dual $\alpha_4\beta_1/\alpha_4\beta_7$ antagonists has been previously reported (Abraham *et al.*, 2000; Koo *et al.*, 2003). It is possible that the engagement of α_4-integrins with VCAM-1 and other activation signals on eosinophils triggers their degranulation. In fact, treatment with an α_4-integrin mAb, HP1/2, reduced antigen-, platelet activating factor- and IL-5-induced eosinophil peroxidase release (Abraham *et al.*, 1994; Milne & Piper, 1995; Kraneveld *et al.*, 1997).

Also relevant is the decrease of protein content in BALF of allergen-challenged sensitized animals treated with TR14035. This result is in contrast with a previous report of increased lung plasma leakage after administration of an anti-α_4 mAb in a rat model of pulmonary inflammation (Taylor *et al.*, 1997). However, other studies in humans and guinea-pigs indicate an association between eosinophil accumulation and plasma exudation (Walls *et al.*, 1991; Collins *et al.*, 1993) that is related to the release of a variety of mediators including sulphidopeptide leukotrienes (Goldie & Pedersen, 1995; Kanehiro *et al.*, 2000). Therefore, the antiexudative effect of TR14035 found in our study may be related to the reduced eosinophil infiltration and activation produced by this antagonist in this experimental model.

In the present study, oral administration of TR14035, in addition to promote a clear reduction in eosinophil influx, also decreased the lymphocyte infiltration. This finding is consistent with the inhibition of lung IL-5 mRNA upregulation elicited by TR14035 treatment observed in this study, which is in agreement with the reported decrease in IL-5 mRNA in BALF cells from rats treated with TA-2, an α_4 mAb (Ramos-Barbon *et al.*, 2001). Despite this finding, the dual $\alpha_4\beta_1/\alpha_4\beta_7$ antagonist TR14035 (3 and $10\,\mathrm{mg\,kg^{-1}}$) failed to significantly diminish IL-4 levels in the BALF of antigen-challenged sensitized animals. IL-4 is released from Th2 lymphocytes and macrophages, and also by mast cells following IgE-crosslinking (Yamaguchi *et al.*, 1997), which might explain the lack of effect of TR14035 on mediator release after antigen challenge in our model. Similar observations have been reported for the systemic administration of anti-α_4 mAb in murine models of asthma (Henderson *et al.*, 1997; Kanehiro *et al.*, 2000; 2001).

IL-4 upregulates VCAM-1 *in vitro* and *in vivo* without affecting the expression of ICAM-1 or E-selectin (Yao *et al.*, 1996; Sanz *et al.*, 1998; Hickey *et al.*, 1999). In our study, we have demonstrated by intravital microscopy that oral treatment with TR14035 inhibits IL-4 function since leukocyte adhesion and emigration were nearly abrogated in animals treated with this antagonist.

Collectively, these data provide strong evidence that the α_4 adhesion pathway participates in pathophysiologic responses associated with the prolonged inflammatory events that follow antigen challenge in the allergic Brown Norway rat model. This is the first report that shows that oral treatment with a dual $\alpha_4\beta_1/\alpha_4\beta_7$ antagonist inhibits most of the characteristic features of allergic pulmonary inflammation. Thus, these results support the potential benefit of antiadhesion therapy in the treatment of pulmonary allergic diseases such as asthma.

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