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Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry 14 (2006) 4208–4216

Selection of a 2-azabicyclo[2.2.2]octane-based $\alpha_4\beta_1$ integrin antagonist as an inhaled anti-asthmatic agent

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Received 16 December 2005; revised 25 January 2006; accepted 26 January 2006
Available online 21 February 2006

Abstract—The $\alpha_4\beta_1$ integrin, expressed on eosinophils and neutrophils, induces inflammation in the lung by facilitating cellular infiltration and activation. From a number of potent $\alpha_4\beta_1$ antagonists that we evaluated for safety and efficacy, **1** was selected as a lead candidate for anti-asthma therapy by the inhalation route. We devised an optimized stereoselective synthesis to facilitate the preparation of a sufficiently large quantity of **1** for assessment in vivo. Administration of **1** to allergen-sensitive sheep by inhalation blocked the late-phase response of asthma and abolished airway hyper-responsiveness at 24 h following the antigen challenge. Additionally, the recruitment of inflammatory cells into the lungs was inhibited. Administration of **1** to ovalbumin-sensitized guinea pigs intraperitoneally blocked airway resistance and inhibited the recruitment of inflammatory cells.

1. Introduction

Integrins are heterodimeric glycoproteins, composed of an α and a β subunit, which are expressed on cell surfaces and involved in cell–cell and cell–extracellular matrix interactions. Two integrins containing the α_4 subunit (CD49D) have been described: $\alpha_4\beta_1$ and $\alpha_4\beta_7$. The integrin $\alpha_4\beta_1$ (very late antigen-4, VLA-4, CD49d/CD29) is expressed on eosinophils, mononuclear leukocytes, mast cells, macrophages, basophils, and neutrophils.[1] In contrast to the prototypical integrins, such as $\alpha_5\beta_1$, $\alpha_{IIb}\beta_{IIIa}$, and $\alpha_v\beta_3$, that recognize the Arg-Gly-Asp (RGD) peptide sequence in their respective protein ligands, $\alpha_4\beta_1$ binds to other primary protein sequences: Gln-Ile-Asp-Ser (QIDS) in vascular cell adhesion molecule-1 (VCAM-1) and Ile-Leu-Asp-Val (ILDV) in fibronectin. Although these $\alpha_4\beta_1$ recognition motifs share a common Asp (D) residue with RGD, they are otherwise unrelated. The $\alpha_4\beta_1$ integrin mediates cell adhesion by binding to either of two ligands, VCAM-1 or the alternatively spliced CS1-containing fibronectin variant, Fn-CS1.[2] Additionally, $\alpha_4\beta_1$ interacts with the matrix ligand osteopontin,[3] which may be important in that osteopontin is strongly up-regulated in inflammatory settings.[4]

The interaction of $\alpha_4\beta_1$ with VCAM-1 is responsible for mononuclear leukocyte and eosinophil adhesion to the endothelium and subsequent transendothelial migration. Integrin $\alpha_4\beta_1$ is believed to mediate airway inflammation by facilitating infiltration of lymphocytes and eosinophils into the lung and inducing expression of inflammatory mediators. Airway eosinophilia is associated with pulmonary inflammation. Moreover, the interaction of $\alpha_4\beta_1$ with its ligands leads to the activation of eosinophils, mast cells, and T-cells, as well as inhibition of apoptosis to increase cell survival.[5] While eosinophil recruitment appears to be involved in the early- and late-phase responses of asthma, conflicting evidence exists as to whether eosinophil recruitment plays a role in airway hyper-responsiveness. For example, in allergen-sensitized sheep and rats,[6,7] inhibition of airway hyper-responsiveness does not correlate to eosinophil

Keywords: Integrin; Antagonist; VLA-4.
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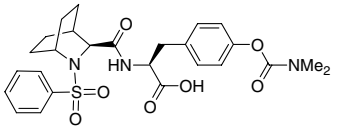
doi:10.1016/j.bmc.2006.01.067

accumulation in broncho-alveolar lavage (BAL) fluid. The identification of $\alpha_4\beta_1$ on neutrophils, particularly those in the lungs, suggests that neutrophils may be a key effector cell in airway hyper-responsiveness.[1d,8] This viewpoint is further supported by the ability of intranasally delivered $\alpha_4\beta_1$ monoclonal antibodies to bind pulmonary neutrophils and block inflammatory responses, such as cytokine release and mucus secretion. Taken together, there is ample evidence that $\alpha_4\beta_1$ is a major player in inflammatory processes in the allergen-sensitized lung via its capability to invoke key cellular and biochemical signals by interacting with its ligands.

Most importantly, antibodies to $\alpha_4\beta_1$ and small-molecule $\alpha_4\beta_1$ antagonists have been reported to inhibit cell recruitment to the lungs and allergic airway responses in animal models of asthma.[6,9–12]

Since $\alpha_4\beta_1$ plays a key role in the migration of leukocytes into tissues during inflammatory responses,[13] an antagonist would be expected to have a therapeutic benefit in a number of inflammatory and autoimmune diseases.[14] Natalizumab, a humanized monoclonal antibody to $\alpha_4\beta_1$, was approved by the FDA for treatment of the autoimmune disease multiple sclerosis and was in clinical trials for treatment of Crohn's disease. However, its marketing and clinical trials have been suspended due to cases of progressive multifocal leukoencephalopathy (PML). The extent of risk is unclear for this highly efficacious drug and is currently under extensive debate and review.[15]

We have been interested in developing a suitable $\alpha_4\beta_1$ antagonist for inhaled anti-asthma treatment, especially to avoid extensive systemic exposure. Previously, we described 2-azabicyclo[2.2.2]octane and 1,2,4-triazolo[2,3-*a*]pyrrole derivatives as novel antagonists of the integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$.[16,17] From the former series, we selected a lead compound that was advanced into in vivo evaluation. In this paper, we present details relating to the synthesis, biological profile, and selection criteria for this advanced lead, 2-azabicyclo[2.2.2]octane derivative **1**. In particular, we report the in vivo evaluation of **1** in sheep and guinea pig models of asthma.



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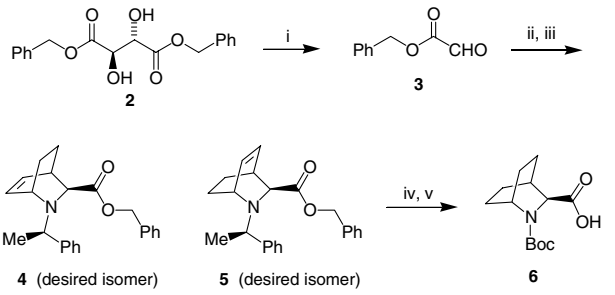
2. Chemistry

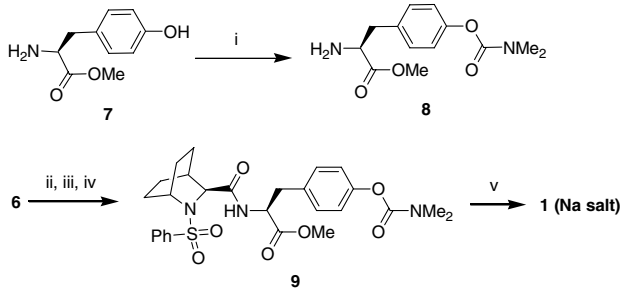
The optimized stereoselective synthesis of the 2,2,2-azabicyclic core is described in this report (Scheme 1). The synthesis of **4** and **5** was realized by optimizing the literature procedure for reaction scale-up.[18] Oxidative cleavage of diol **2** by using periodic acid provided benzylglyoxylate **3**. Treatment of benzylglyoxylate **3** with (*R*)-α-methylbenzyl amine and 4 Å molecular sieves in CH_2Cl_2 at 3 °C followed by gradually warming to



Scheme 1. Synthesis of **6**. Reagents and conditions: (i) HIO_6, Et_2O; (ii) (*R*)-(+)-α-methylbenzylamine, 4 Å MS, CH_2Cl_2, 3 to 23 °C; (iii) CF_3CO_2H, BF_3-OEt_2, 1,3-cyclohexadiene, −25 °C, 74% **4** + **5**; (iv) 10% Pd/C, H_2O, MeOH, 50 psig H_2, 85%; (v) Boc_2O, 3 N NaOH, 1,4-dioxane, 0–23 °C, 99%.

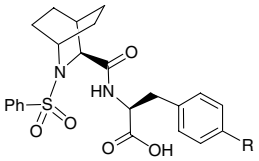
room temperature over 18 h formed the corresponding imine in situ. The formation of the imine was achieved more effectively by this method versus using a 30-min time period at 0 °C, as reported in the literature. The molecular sieves were removed and the solution was cooled to −25 °C. Trifluoroacetic acid, boron trifluoride etherate, and 1,3-cyclohexadiene were added sequentially followed by stirring at −25 °C for 16 h to provide desired diastereomers **4** and **5** in 74% overall yield after chromatography. When the Diels–Alder reaction was performed according to the literature[18] without removing the molecular sieves and at −78 °C for 5 h followed by warming to room temperature, diastereomers **4** and **5** were isolated in only 38% overall yield after chromatography. Hydrogenation of **4** and **5** reduced the alkene, removed the benzyl ester, and cleaved the α-methylbenzyl group to give the corresponding 2,2,2-bicyclic amino acid in good yield as a single isomer. Treatment of this amino acid with *t*-butoxycarbonylanhydride gave Boc-protected amino acid **6**.

We synthesized dimethylcarbamate derivative **1** starting with amino ester **8**, which was obtained in good yield by deprotonation of L-tyrosine methyl ester with NaH followed by addition of dimethylcarbamoyl chloride at −15 °C (Scheme 2). Carboxylic acid **6** was coupled with amine **8** by using bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl) to yield the *N*-Boc-protected 2,2,2-azabicyclic amide. Removal of the Boc-protecting group with trifluoroacetic acid, followed by sulfonamide for-



Scheme 2. Synthesis of target compound **1**. Reagents and conditions: (i) NaH, dimethylcarbamoyl chloride, DMSO, THF, −15 to 0 °C, 56%; (ii) **8**, BOP-Cl, Et_3N, CH_2Cl_2, 60%; (iii) CF_3CO_2H, CH_2Cl_2, 97%; (iv) $PhSO_2Cl$, Et_3N, CH_2Cl_2, 0 to 23 °C, 98%; (v) NaOH, H_2O, THF, 99%.

Table 1. Inhibition binding of $\alpha_4\beta_1$ (Ramos) or $\alpha_4\beta_7$ (K562) positive cells to hVCAM-1 by N-phenylsulfonyl[2.2.2]bicyclooctane derivatives (IC_{50})



Compound	R group	$\alpha_4\beta_1$ (nM)	$\alpha_4\beta_7$ (nM)
10	–NHC(O)-4-pyridyl	36 ± 9	120 ± 40
11	–NHC(O)-2,6-dichloro-4-pyridyl	12 ± 4	46 ± 9
12	Phthalimido	26 ± 3	110 ± 40
1	$-OC(O)NMe_2$	39 ± 9	440 ± 150

mation with benzenesulfonyl chloride, gave the 2,2,2-azabicyclic derivative **9**. Hydrolysis of the methyl ester with NaOH provided the corresponding sodium salt of **1**. Derivatives **10**–**12** (Table 1) were prepared by similar methods, as described elsewhere.[16]

3. In vitro biological results

Previously, we described the SAR for the 2-azabicyclo-[2.2.2]octane arylsulfonamide series of α_4 integrin antagonists, along with some in vivo results at a single dose for three of the best compounds (Table 1, **10**–**12**).[16a] Here, we present a more detailed pharmacological profile of **1** and our rationale for designating **1** as a lead compound. Compound **1** has good in vitro inhibition against the $\alpha_4\beta_1$ (IC_{50} = 39 nM) integrin with potency similar to that of **10**–**12**. Like **10** and **12**, only modest potency was observed in the $\alpha_4\beta_7$ (IC_{50} = 440 nM) adhesion assay. In contrast, the 2,6-dichloro-4-pyridyl derivative **11** showed good dual potency against the two α_4 integrins. Only weak inhibition for **1** against the $\alpha_5\beta_1$ integrin was observed (IC_{50} = 7200 nM). Compound **1** exhibits high aqueous solubility (>1 mg/mL) at pH 7.4, and excellent stability in human liver microsomes ($t_{1/2}$ > 100 min) and in human and rat hepatic S9 cells (89% and 86% remaining at 90 min, respectively). Human lung S9 fraction also had no effect on **1**, with >99% remaining at 90 min. By contrast, **11** was only moderately stable in human liver microsomes ($t_{1/2}$ = 39 min). Compound **1** showed no inhibition of any P450 enzymes. Reasonable plasma protein binding was observed, 87% in plasma and 91% in serum albumin, and there was low binding to red blood cells (13%).

4. In vivo biological studies

We proceeded to evaluate **1** in the well-characterized preclinical sheep model of asthma[19] via inhalation delivery and compare it to **10**–**12**.[16a] The allergic sheep model measures the efficacy of experimental compounds against three distinct responses to allergen provocation.[19] Aerosol challenge of naturally sensitive animals with the antigen *Ascaris suum* results in an immediate increase in specific lung resistance (SR_L), known as the early-phase response. Between 4 and 6 h after antigen challenge, SR_L returns to baseline, but then 6–8 h following antigen challenge, SR_L is elevated a second time, resulting in a late-phase broncho-constrictive response. Airway hyper-responsiveness is observed at 24 h following antigen challenge. These three responses in the allergic sheep model parallel the airway responses that are seen in humans with asthma. In a preliminary study (Fig. 1), a single aerosolized dose of **1** (0.25 mg/kg) was administered, 30 min prior to antigen challenge. A modest effect was seen on airway resistance in the early-phase and the late-phase was completely blocked, similar to what was observed with **12**.[16a] All four compounds blocked airway hyper-responsiveness, but **10** and **11** had no effect on the early phase of airway resistance. Although **1** and **12** exhibited the best efficacy in the initial asthma model, **12** possessed some cardiovascular liabilities.[20] Thus, **1** was selected for further evaluation.

Compound **1** was then evaluated in the sheep asthma model at two doses, 0.075 and 0.25 mg/kg, administered twice daily for three consecutive days and 0.5 h prior to antigen challenge via inhalation. The early-phase response was slightly affected only by the lower dose, while the late-phase response was completely blocked by either dose of **1**. The airway hyper-responsiveness was completely abolished with the 0.25 mg/kg dose, while the 0.075 mg/kg dose was partially effective (Fig. 2).

Broncho-alveolar lavage (BAL) fluid from antigen-treated sheep was also examined for changes in cell influx at baseline (prior to antigen) and at 8 and 24 h after antigen challenge at both doses of **1**. A pronounced reduction of eosinophil recruitment was observed at 0.075 and 0.25 mg/kg, at both the 8 and 24 h time points (Fig. 3). Neutrophil recruitment was also markedly reduced at 24 h for both doses relative to the vehicle group. Compound **1** showed moderate bioavailability by the inhalation route in guinea pigs (F = 9%, C_{max} of 1.5 μM, $t_{1/2}$ = 0.8 h, 10 mg/kg) and was rapidly eliminated from the plasma. It was not orally bioavailable in rats (F < 1%).

To demonstrate the efficacy of **1**, a second species, we used the passively sensitized guinea pig model,[21] assessing airway hyper-responsiveness and eosinophil recruitment into the BAL fluid. Aerosol challenge of passively sensitized guinea pigs with ovalbumin (OVA) for 1 h produced a marked increase in the airway hyper-responsiveness at 24 h relative to a naïve group. A large increase of eosinophil recruitment was observed in the BAL fluid at the 24 h time point. Compound **1** was administered intraperitoneally for 7 days and at 24 h prior to assessing lung function in the passively sensitized guinea pigs at three different doses (0.1, 0.5, and 1 mg/kg). Examination of the BAL fluid showed a significant reduction in eosinophil recruitment at the doses of 0.5 and 1 mg/kg (Fig. 4). A reduction in eosinophil recruitment was also observed at 0.1 mg/kg, but to a lesser extent. Airway hyper-responsiveness at 24 h

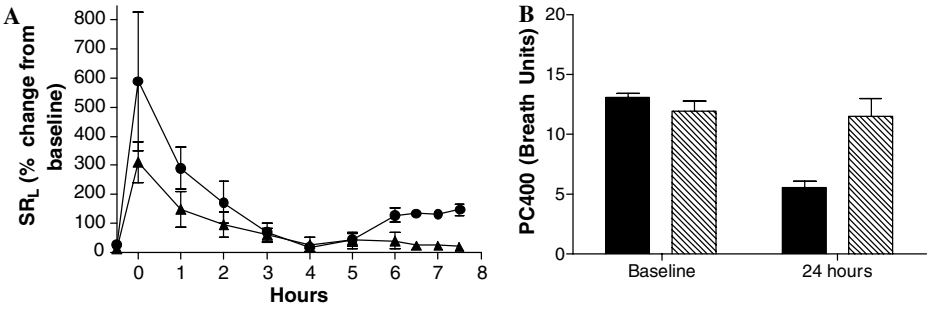


Figure 1. Effect of aerosol administration of **1** to conscious allergic sheep on airway mechanics. (A) Increase in SR_L in control animals (●) and animals treated with **1** (▲), single dose of 0.25 mg/kg, 0.5 h prior to antigen challenge. (B) Change in airway responsiveness at baseline and 24 h post-antigen challenge in animals treated with single 0.25 mg/kg dose of **1** (hatched bar) and control animals (solid bar).

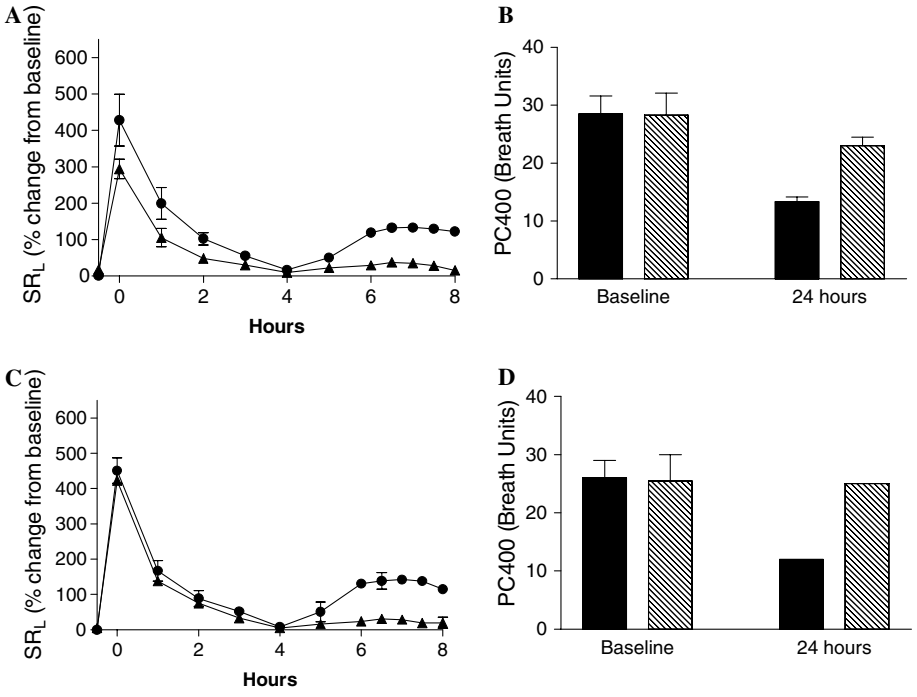


Figure 2. Effect of aerosol administration of **1** to conscious allergic sheep by aerosol on airway mechanics. (A) Increase in SR_L in control animals (●) and animals treated with **1** (▲) at 0.075 mg/kg. (B) Change in airway responsiveness at baseline and 24 h post-antigen challenge in animals treated with 0.075 mg/kg of **1**; solid bar, control animals; hatched bar animals treated with **1**. (C) Increase in SR_L in control animals (●) and animals treated with **1** (▲) at 0.25 mg/kg. (D) Change in airway responsiveness at baseline and 24 h post-antigen challenge in animals treated with 0.25 mg/kg of **1**; solid bar, control animals; hatched bar, treated animals.

showed a large reduction at the 0.5-mg/kg dose with respect to the increase in airway resistance resulting from ovalbumin treatment. In contrast, when **1** was administered at 0.1 mg/kg, there was no effect on airway hyperresponsiveness at 24 h.

5. Conclusion

Compound **1** was selected as our lead candidate for an inhaled anti-asthma therapeutic, because of its good potency in the $\alpha_4\beta_1$ adhesion assay (IC_{50} = 39 nM), its clean in vitro and in vivo safety profile, and its excellent efficacy in the sheep model of asthma. When dosed by inhalation to allergen-sensitive sheep, **1** blocked the late-phase asthmatic response, abolished airway hyperresponsiveness at 24 h post-dosing, and caused a marked reduction in eosinophil and neutrophil cell recruitment to the lungs. In guinea pigs, **1** produced a large reduction in inflammatory cell recruitment, which is consistent with antagonism of $\alpha_4\beta_1$.

6. Experimental

6.1. General methods

^{1}H NMR spectra were acquired at 300.14 MHz on a Bruker Avance-300 spectrometer in $CDCl_3$ unless indicated otherwise, using Me_4Si as an internal standard. NMR abbreviations used: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet; br, broad; ov, overlapping. HPLC analyses were performed on a Hewlett Packard Series 1100 HPLC instrument eluting with

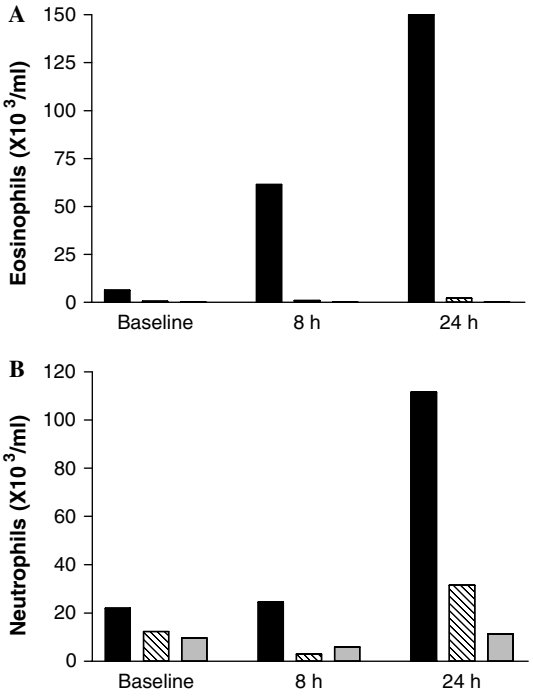


Figure 3. Effect of aerosol administration of **1** to conscious allergic sheep by aerosol on cell influx to the BAL. (A) Number of eosinophils in BAL at baseline, 8 and 24 h after antigen challenge. Solid bar represents BAL from control animals; hatched bar represents BAL from animals treated with 0.075 mg/kg of **1**; gray bar represents animals treated with 0.25 mg/kg of **1** in both graphs. (B) Number of neutrophils in BAL at baseline, 8 and 24 h after antigen challenge.

a gradient of water/MeCN/CF_3CO_2H (10:90:0.2 to 90:10:0.2) over 4 min with a flow rate of 0.75 mL/min on a Kromasil C18 column (50 × 2.0 mm; 3.5 μm particle size) or on a Supelcosil ABZ+Plus column (50 × 2.0 mm; 3.5 μm particle size) at 32 °C. Signals were recorded simultaneously at 220 and 254 with a diode array detector. Normal-phase preparative chromatography was performed on an Isco Combiflash Separation System Sg 100c equipped with a Biotage FLASH Si 40M silica gel cartridge (KP-Sil Silica, 32–63 μ, 60 Å; 4 × 15 cm) eluting at 35 mL/min with detection at 254 nm. Reversed-phase preparative chromatography was performed on a Gilson HPLC with a Kromasil column (10 μ, 100 Å C18, column length 250 × 50 mm). Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Electrospray (ES) mass spectra were obtained on a Micromass Platform LC single quadrupole mass spectrometer in the positive mode. Accurate mass spectra were run on a Micromass Autospec-OA-TOF double focusing mass spectrometer using fast atom bombardment ionization with thioglycerol as the sample matrix. Elemental analysis and Karl Fischer water analysis were determined by Quantitative Technologies Inc., Whitehouse, NJ.

6.2. 2-(1-(*S*)-Phenethyl)-2-(*S*)-azabicyclo[2.2.2]oct-5-ene-3-carboxylic acid benzyl ester (4 and 5)

A 3-L, one-necked round-bottomed flask (equipped with nitrogen inlet) was charged with dibenzyl tartrate

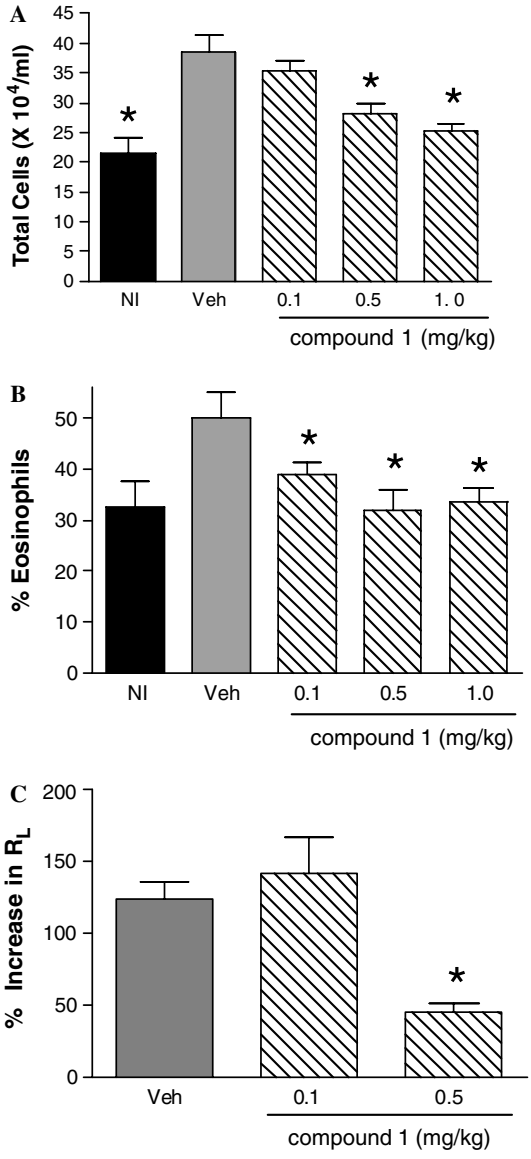


Figure 4. Effect of ip administration of **1** to passively sensitized guinea pigs. (A) Total cell number in BAL collected at 24 h following antigen challenge. Solid bars represent non-immunized animals (NI); gray bars represent immunized vehicle (Veh)-treated animals; hatched bars represent animals treated with 0.1, 0.5, and 1 mg/kg of **1**. (B) Percent eosinophils of total cells recovered from BAL collected at 24 h following antigen challenge. Bars have the same meaning as in previous graph. (C) Percent increase in R_L following challenge with OVA (100 μg/kg) at 24 h post-initial exposure to OVA. *Significance of $P < 0.05$, ANOVA; $N = 6$.

(**2**, 86.5 g, 0.262 mol) and 2 L of anhydrous ethyl ether. To the resulting solution was added periodic acid dihydrate (59.7 g, 0.262 mol). The slurry was stirred at 23 °C for 1.5 h during which a white precipitate had formed. The cloudy solution was filtered through Celite (washing with 200 mL ethyl ether) and the filtrate was concentrated in vacuo (not to dryness) to give benzyl glyoxylate (**3**), which was used without any further purification. ^1H NMR (C_6D_6) δ 8.87 (s, 1H), 7.28–7.43 (m, 5H), 5.12 (s, 2H). The crude benzyl glyoxylate (85.9 g, 0.524 mol) was dissolved in dichloromethane (2 L) and charged into a 3-L,

four-necked flask (equipped with mechanical stirrer, nitrogen inlet, thermocouple, and a glass stopper). The reaction was cooled in an ice water bath to an internal temperature of ~3 °C and was treated sequentially with 104 g of freshly crushed 4 Å molecular sieves and (*R*)-(+)-α-methyl benzylamine (62.2 g, 0.514 mol). The reaction mixture was allowed to slowly warm to room temperature over an 18 h period (overnight). The molecular sieves were removed by filtration through Celite (washing with dichloromethane) and the filtrate of the desired imine was used directly without any purification. The imine solution (~0.524 mol) was charged into a 5-L, three-necked flask (equipped with a mechanical stirrer, nitrogen inlet, and a thermocouple), diluted with dichloromethane (500 mL), and chilled in a dry ice/acetonitrile bath to an internal temperature of −25 °C. To this solution were added sequentially trifluoroacetic acid (59.7 g, 0.524 mol), boron trifluoride etherate (74.4 g, 0.524 mol), and 1,3-cyclohexadiene (46.2 g, 0.576 mol). The reaction mixture was stirred for 5 h keeping the internal temperature between −25 and −15 °C by occasional addition of additional dry ice. The reaction appeared to be mostly complete by NMR (disappearance of imine C-H) but the reaction mixture was stored in freezer (−25 °C) for 16 h (overnight). The reaction mixture was split into two equal portions and each portion was quenched into 1.5 L of saturated sodium bicarbonate and stirred vigorously for 1.5 h. The layers were separated and the aqueous phase was extracted with dichloromethane (2 × 250 mL). The organic extracts were each washed with saturated sodium bicarbonate (250 mL) then combined, dried ($MgSO_4$), and concentrated in vacuo to give 208 g of crude product. The red oil was dissolved in ethyl acetate and passed through a Biotage-activated carbon cartridge (75M, ~400 g), and eluted off with ethyl acetate (3 L). This material was dissolved in heptane and dichloromethane (1 L: 50 mL), and loaded onto a Biotage 150 L (5 kg silica gel) and eluted with heptane (4 L), and then ethyl acetate–heptane 3:97 (36 L), 1:19 (20 L), 1:9 (10 L), and 1:4 (20 L) to yield 101.7 g (57%) of **4** and 30.3 g (17%) of **5**.

Compounnd **4**: $[\alpha]_D$ −64.6° (*c* 1.23, $CHCl_3$, 23 °C); one isomer by ^1H NMR ($CDCl_3$) δ7.16–7.40 (m, 10H), 6.37–6.41 (m, 1H), 6.25–6.30 (m, 1H), 4.94 (s, 2H), 3.60–3.66 (m, 1H), 3.40–3.47 (m, 1H), 2.92–2.98 (m, 1H), 2.73–2.76 (m, 1H), 2.01–2.06 (m, 2H), 1.52–1.63 (m, 3H), 1.21–1.33 (m, 5H), 1.01–1.06 (m, 1H), 0.86–0.90 (m, 1H); MS (ES) *m*/*z* = 348 $(M+H)^+$; HRMS (FAB) *m*/*z* 348.1979 (348.1964 calcd for $C_{13}H_{21}NO_4 + H^+$).

Compound **5**: $[\alpha]_D$ −13.5° (*c* 1.26, $CHCl_3$, 23 °C); one isomer by ^1H NMR ($CDCl_3$) δ 7.14–7.36 (m, 8H), 7.04–7.07 (m, 2H), 6.65–6.71 (m, 1H), 6.08–6.13 (m, 1H), 4.49–4.69 (m, 2H), 3.93–3.96 (m, 1H), 3.59–3.63 (m, 1H), 3.07–3.11 (m, 1H), 2.66–2.68 (m, 1H), 2.08–2.12 (m, 2H), 1.61–1.65 (m, 3H), 1.32–1.59 (m, 5H), 1.11–1.26 (m, 1H), 0.85–0.88 (m, 1H); MS (ES) *m*/*z* = 348 $(M+H)^+$; HRMS (FAB) *m*/*z* 348.1964 (348.1964 calcd for $C_{13}H_{21}NO_4 + H^+$).

6.3. (*S*)-2-Azabicyclo[2.2.2]octane-3-carboxylic acid[18]

A 2-L Parr bottle was charged (under nitrogen) with 10% palladium on carbon, ~50% water (18 g, Degussa type E101 NE/W), and methanol (250 mL). The 2-(1-(*S*)-phenethyl)-2-(*S*)-azabicyclo[2.2.2]oct-5-ene-3-carboxylic acid benzyl ester (**4** and **5**, 88.4 g, 0.254 mol) was dissolved in methanol (250 mL), added the Parr bottle, and diluted with methanol (500 mL). The reaction mixture was agitated on a shaker at 23 °C under 40 psig of hydrogen for 2 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo to give a semi-solid product, which was dissolved in ethyl acetate (200 mL), with a minimal amount of methanol, and treated with 1 N HCl in ethyl ether (275 mL). The mixture was concentrated until a slurry of white solid formed, diluted with ethyl acetate (100 mL), and concentrated again. This process was repeated two more times to remove methanol, and the resulting off-white solid was collected by filtration/washing with ethyl acetate (200 mL) and ethyl ether (100 mL). After drying, 41.2 g (85%) of the desired amino acid hydrochloride salt was obtained as an off-white solid.

$[\alpha]_D$ +14.2° (*c* 2.15, MeOH; 23 °C), literature $[\alpha]_D$ +15.0° (*c* 0.85, EtOH);[18] ^1H NMR (CD_3OD) δ 4.13–4.16 (m, 1H), 3.49–3.53 (m, 1H), 2.33–2.37 (m, 1H), 1.74–1.90 (m, 9H); MS (ES) *m*/*z* = 156 $(M + H)^+$. Anal. Calcd for $C_8H_{13}NO_2 \cdot HCl$: C, 50.14; H, 7.36; N, 7.31; Cl, 18.50. Found: C, 50.37; H, 7.63; N, 7.13; Cl, 18.35.

6.4. (*S*)-2-*N*-(*t*-Butyloxycarbonyl)azabicyclo[2.2.2]-octane-3-carboxylic acid (6)

A 1-L round-bottomed flask containing (*S*)-2-azabicyclo-[2.2.2]octane-3-carboxylic acid (11.34 g, 59.4 mmol) and 1,4-dioxane (300 mL) was cooled in an ice/water bath. 3 N NaOH (60 mL, 180 mmol) was added, followed by di-*t*-butyl dicarbonate (13.02 g, 59.7 mmol), and the mixture was stirred at 0 °C for 1 h, and then at 23 °C for 4 h. After quenching with citric acid (34.7 g, 181 mmol), the mixture was diluted with water (200 mL) and extracted with ethyl acetate (2 × 600 mL). The combined organic layers were dried ($MgSO_4$), filtered through Celite®, and concentrated in vacuo to give 15.0 g (99%) of **6** as a white solid.

$[\alpha]_D$ −29.2° (*c* 1.11, MeOH; 23 °C); ^1H NMR (CD_3OD) δ 4.12–4.14 (m, 1H), 3.95–4.02 (m, 1H), 2.12–2.19 (m, 1H), 1.89–1.98 (m, 1H), 1.47–1.77 (m, 7H), 1.42 (s, 9H); HRMS (FAB) *m*/*z* 256.1550 (256.1549 calcd for $C_{13}H_{21}NO_4 + H^+$).

6.5. Methyl 2-(*S*)-amino-3-(4-dimethylcarbamoyloxyphenyl)propanoate (8)

A 1-L round-bottomed flask containing L-tyrosine methyl ester (21.0 g, 0.11 mol), tetrahydrofuran (540 mL), and dimethylsulfoxide (42 mL, 0.59 mol) was cooled to 0 °C with a dry ice/acetone bath. Sodium hydride (3.0 g, 0.13 mol, 95%) was added in 4 equal portions over 15 min. The mixture was stirred in the dry ice/acetone bath at 0 °C until all hydrogen evolution ceases,

cooled to −20 °C, and treated with dimethylcarbamoyl chloride (9.9 mL, 0.11 mol), added gradually over 5 min. The mixture was stirred at −15 °C for 30 min and at 0 °C for 3 h. The mixture was quenched with 1 N NaOH (350 mL), transferred to a separatory funnel, and extracted with dichloromethane (2× 500 mL). The organic layers were combined, dried ($MgSO_4$), filtered through Celite®, and concentrated in vacuo to give 16.0 g (56%) of **8** as a white solid. This compound is not stable at 23 °C over time and was used without further purification.

^{1}H NMR (CD_3OD) δ 7.30 (d, J = 8.4 Hz, 2H), 7.12 (d, J = 8.4 Hz, 2H), 4.31 (t, J = 6.6 Hz, 1H), 3.84 (s, 3H), 3.27–3.36 (m, 2H), 3.13 (s, 3H), 3.01 (s, 3H); MS (ES) m/z = 267 $(M+H)^+$.

6.6. Methyl 2-(*S*)-[(2-(*S*)-*N*-(*t*-butyloxycarbonyl)aza-bicyclo[2.2.2]octane-3-carbonyl)amino]-3-(4-dimethylcarbamoyloxyphenyl)propanoate

A 500-mL round-bottomed flask containing **6** (15.2 g, 59.6 mmol), dichloromethane (200 mL), and triethylamine (17.0 mL, 122 mmol) was cooled with an ice/water bath. Bis(2-oxo-3-oxazolidinyl)phosphinic chloride (23.4 g, 91.9 mmol) was added and the mixture was stirred in the ice/water bath for 15 min. Compound **8** (16.0 g, 60.2 mmol) was added via cannulation in dichloromethane (100 mL). The mixture was stirred in the ice/water bath for 1 h and then at 23 °C for 16 h. The mixture was concentrated in vacuo and purified by flash chromatography (230–400 mesh silica gel 60, 98:2 dichloromethane/MeOH) to give 17.9 g (60%) of methyl 2-(*S*)-[(2-(*S*)-*N*-Boc-azabicyclo[2.2.2]octane-3-carbonyl)amino]-3-(4-dimethylcarbamoyloxyphenyl)-propanoate as a white solid.

$[\alpha]_D$ −28.5° (*c* 1.11, MeOH, 23 °C); ^{1}H NMR (CD_3OD) δ 7.26 (d, J = 7.8 Hz, 2H), 7.01 (d, J = 8.4 Hz, 2H), 4.75–4.81 (m, 1H), 4.02–4.06 (m, 1H), 3.95–4.00 (m, 1H), 3.71 (s, 3H), 3.17–3.23 (m, 2H), 3.10 (s, 3H), 2.98 (s, 3H), 2.05–2.09 (m, 1H), 1.46–1.77 (m, 8H), 1.32 (s, 9H); HRMS (FAB) m/z 504.2716 (504.2710 calcd for $C_{26}H_{37}N_3O_7$+H^+).

6.7. Methyl 2-(*S*)-[(2-(*S*)-azabicyclo[2.2.2]octane-3-carbonyl)amino]-3-(4-dimethylcarbamoyloxyphenyl)-propanoate, trifluoroacetic acid salt

A 500-mL round-bottomed flask containing methyl 2-[(2-(*S*)-*N*-Boc-azabicyclo[2.2.2]octane-3-carbonyl)amino]-3-(*S*)-(4-dimethylcarbamoyloxyphenyl)propanoate (11.0 g, 21.9 mmol) and dichloromethane (110 mL) was treated with trifluoroacetic acid (20 mL) and then stirred at 23 °C for 1 h. The mixture was concentrated in vacuo to give 11.0 g (97%) of methyl 2-(*S*)-[(2-(*S*)-azabicyclo[2.2.2]octane-3-carbonyl)amino]-3-(4-dimethylcarbamoyloxyphenyl)-propanoate trifluoroacetic acid salt as a white solid.

^{1}H NMR (CD_3OD) δ 7.23 (d, J = 8.5 Hz, 2H), 7.02 (d, J = 8.6 Hz, 2H), 4.76–4.80 (m, 1H), 3.88–3.98 (m, 1H), 3.73 (s, 3H), 3.31–3.45 (m, 2H), 3.10 (s, 3H), 2.98 (s, 3H), 2.23–2.27 (m, 1H), 1.66–1.83 (m, 8H). Anal. Calcd for $C_{21}H_{29}N_3O_5{\cdot}1.4C_2HF_3O_2{\cdot}0.4H_2O$: C, 50.12; H, 5.51; N, 7.37; F, 13.99; H_2O, 1.26. Found: C, 50.47; H, 5.21; N, 7.20; F, 14.09; H_2O, 1.30.

6.8. Methyl 2-(*S*)-[(2-benzenesulfonyl-2-(*S*)-azabicyclo-[2.2.2]octane-3-carbonyl)amino]-3-(4-dimethyl-carbamoyloxyphenyl)propanoate (9)

A 200-mL round-bottomed flask containing methyl 2-[(2-(*S*)-azabicyclo[2.2.2]octane-3-carbonyl)amino]-3-(*S*)-(4-dimethylcarbamoyloxyphenyl)propanoate trifluoroacetic acid salt (11.0 g, 21.3 mmol) and dichloromethane (110 mL) was cooled in an ice/water bath. Triethylamine (7.4 mL, 53.1 mmol) was added followed by benzenesulfonyl chloride (3.0 mL, 23.5 mmol). The mixture was stirred in the bath for 30 min, and at 23 °C for 2 h, then placed in a separatory funnel, and diluted with dichloromethane (400 mL). The organic phase was washed with 1 N HCl (200 mL) and 1 N NaOH (200 mL). The organic layer was dried ($MgSO_4$), filtered through Celite®, and concentrated in vacuo. The product was purified via flash chromatography (230–400 mesh silica gel 60, 99:1 dichloromethane/MeOH) to give 11.4 g (98%) of **9** as a white solid.

$[\alpha]_D$ −82.9° (*c* 1.04, $CHCl_3$; 23 °C); ^{1}H NMR ($CDCl_3$) δ 7.91 (d, J = 7.2 Hz, 2H), 7.60–7.62 (m, 1H), 7.48–7.58 (m, 2H), 7.15 (d, J = 6.4 Hz, 2H), 7.02 (d, J = 8.6 Hz, 2H), 4.88–4.91 (m, 1H), 4.00–4.03 (m, 1H), 3.76 (s, 3H), 3.19–3.22 (m, 2H), 3.08 (s, 3H), 2.99 (s, 3H), 2.20–2.25 (m, 1H), 1.93–1.98 (m, 1H), 1.42–1.59 (m, 4H), 1.13–1.28 (m, 4H). Anal. Calcd for $C_{27}H_{33}N_3O_7S{\cdot}0.45H_2O$: C, 58.78; H, 6.19; N, 7.62; H_2O, 1.47. Found: C, 58.46; H, 6.16; N, 7.66; H_2O, 1.35.

6.9. 2-(*S*)-[(2-Benzenesulfonyl-2-(*S*)-azabicyclo[2.2.2]-octane-3-carbonyl)amino]-3-(4-dimethylcarbamoyl-oxyphenyl)propanoic acid, sodium salt (1-Na)

A 500-mL round-bottomed flask containing **9** (11.79 g, 21.9 mmol) and tetrahydrofuran (110 mL) was treated with a solution of NaOH (0.90 g, 22.5 mmol) in water (210 mL), added by addition funnel. The mixture was stirred at 23 °C for 30 min and concentrated in vacuo. The product was precipitated from 2-propanol/diethyl ether to give 11.8 g (99%) of **1** as a white solid.

$[\alpha]_D$ −42.7° (*c* 1.53, CH_3OH; 23 °C); ^{1}H NMR ($CDCl_3$) δ 7.97 (d, J = 7.2 Hz, 2H), 7.58–7.68 (m, 3H), 7.29 (d, J = 8.4 Hz, 2H), 6.96 (d, J = 8.4 Hz, 2H), 4.41–4.44 (m, 1H), 4.02–4.06 (m, 1H), 3.68–3.72 (m, 1H), 3.16–3.33 (m, 2H), 3.10 (s, 3H), 2.99 (s, 3H), 2.04–2.08 (m, 1H), 1.72–1.80 (m, 1H), 1.51–1.55 (m, 4H), 1.20–1.29 (m, 4H); MS (ES) m/z = 551 $(M+Na)^+$; Anal. Calcd for $C_{26}H_{30}N_3O_7S{\cdot}Na{\cdot}2.2H_2O$: C, 52.82; H, 5.86; N, 7.11; H_2O, 6.70. Found: C, 52.61; H, 5.62; N, 7.03; H_2O, 6.88.

6.10. Adhesion of ramos cells ($\alpha_4\beta_1$ specific) to VCAM-1

This adhesion assay was modified from that reported by Jackson et al.[22] Ultrahigh binding 96-well plates (Dynex

Technologies, Chantilly, VA) were coated with 100 μL recombinant hVCAM-1 at 4.0 μg/mL in 0.05 M $NaCO_3$ buffer, pH 9.0, overnight at 4 °C(R&D Systems). Plates were washed twice in calcium- and magnesium-free PBS with 1% BSA and blocked for 1 h at 23 °C in this buffer. PBS was removed and compounds (50 μL) were added at 2× concentration. Dose–responses were then determined in duplicate. Ramos cells (ATCC, Manassas, VA), 50 μL at 2×10^6 cells/mL, labeled with 5 μM Calcein AM (Molecular Probes) for 30 min at 37 °C, were added to each well and allowed to adhere for 1 h at 23 °C. Plates were washed four times in PBS containing 1% BSA and cells were lysed for 15 min in 100 μL of 1 M Tris–HCl buffer at pH 8.0 with 1% SDS. Plates were read at 485-nm excitation and 530-nm emission on a CytoFluor 4000 fluorescent plate reader (Applied Biosystems, Foster City, CA).

6.11. Adhesion of K562 cells that express $\alpha_4\beta_7$ to VCAM-1

K562 cells expressing human $\alpha_4\beta_7$ were licensed from Dr. David Erle (UCSF).[23] Compounds were evaluated for their ability to inhibit adhesion of $\alpha_4\beta_7$-expressing K562 cells to hVCAM-1 by using identical methods described for Ramos cell adhesion to hVCAM-1.

6.12. Adhesion of K562 cells ($\alpha_5\beta_1$ specific) to human fibronectin

K562 cells (ATCC) possess a single α chain (α_5) and specifically bind to human fibronectin via $\alpha_5\beta_1$. Adhesion assays were carried out similar to the above methods with the exception that human fibronectin (Sigma, St. Louis, MO) at 10 μg/mL was plated onto microtiter plates in 0.05 M Na_2CO_3 buffer, pH 9.0, overnight at 4 °C.[24]

6.13. Antigen-induced airway response in sheep

The validated model of *A. suum* antigen-induced asthmatic response in conscious sheep was used to evaluate the effectiveness of compound **1** in inhibiting antigen-induced early and late airway bronchoconstriction.[6] Animals used in these studies ($N = 4$ per treatment) exhibited both early and late airway responses as well as airway hyper-responsiveness to inhalation challenge with *A. suum* antigen. Briefly, aerosol of *A. suum* extract was reproducibly generated using a disposable medical nebulizer (Raindrop®, Puritan Bennett). The aerosol was delivered at a tidal volume of 500 mL and a rate of 20 breaths/min for 20 min. Compound **1** was administered by aerosol, BID, for 3 consecutive days, and a single dose on day 4, 30 min prior to antigen challenge. Two doses, 3 and 10 mg (ca. 0.075 and 0.25 mg/kg based on an average body weight of 40 kg), were evaluated using this protocol ($N = 3$ sheep at each dose). Analysis of 5–10 breaths, by previously described methods, was used for the determination of R_L, which is calculated by dividing the change in transpulmonary pressure by the change in flow at mid-tidal volume. Immediately after the measurement of R_L, thoracic gas volume (V_{tg}) was determined in a constant volume body plethysmograph to obtain specific lung resistance ($SR_L = R_L \times V_{tg}$). Post-drug measurements of SR_L were obtained immediately prior to challenge with *A. suum* antigen. Measurements of SR_L were obtained immediately after challenge, hourly from 1 to 6 h after challenge and on the half-hour from 6.5, 5–8 h after challenge. Measurements of SR_L were obtained 24 h after challenge, followed by the 24 h post-challenge dose–response curve for carbachol. The results for these studies are compared to each sheep's historical control.

To determine the effect of **1** on the hyper-reactivity stage, measurements of SR_L were repeated immediately after inhalation of buffer and after each administration of 10 breaths of increasing concentrations of carbachol solution (0.25%, 0.5%, 1.0%, 2.0%, and 4.0% wt/vol). To assess airway responsiveness, the cumulative carbachol dose in breath units (BU) that increases SR_L 400% over the post-buffer value (i.e., PC_{400}) was calculated from the dose-response curve. One breath unit is defined as one breath of a 1% wt/vol carbachol solution.

BAL was sampled before antigen challenge, and at 8 and 24 h following antigen challenge, to evaluate cell influx into the lungs. Lung lavage was performed as previously described via infusion and aspiration of 30 mL aliquots of PBS (pH 7.4) at 39 °C.[9]

6.14. Antigen-induced airway resistance and eosinophilia in passively sensitized guinea pigs

Methods were performed as described in previous publications with slight modifications.[21b,25] Briefly, male Dunkin Hartley guinea pigs were immunized ip with a 1.0 mL solution of ovalbumin (OVA) in $Al(OH)_3$ (10 μg OVA per animal), while control animals received $Al(OH)_3$ alone ($N = 5$ per group). This procedure was repeated on day 14. On day 21, blood was collected, centrifuged, and plasma collected. This anti-OVA plasma was injected into naïve guinea pigs (1 mL/animal). In the initial study, seven days later and 24 h prior to assessing lung function, passively sensitized guinea pigs were injected with **1** (dissolved in DMSO; 0.1, 0.5, and 1 mg/kg; ip) or vehicle. Thirty minutes later, animals were exposed for 1 h to aerosolized ovalbumin (dissolved in sterile 0.9% physiological saline, 100 μg/ml) in an exposure chamber in which the guinea pigs could move freely. Twenty-four hours following OVA exposure, animals were anesthetized and the trachea was cannulated and attached to a ventilator (60 breaths/min). The carotid artery was cannulated for the measurement of blood pressure. Changes in airway resistance in response to histamine (1, 2, and 4 μg/kg) and OVA (100 μg/kg) were measured. Data was collected for animals treated with either vehicle or 0.1 and 0.5 mg/kg of **1**. BAL fluid was also collected at 24 h.

To obtain BAL fluid, 5 mL of sterile saline was slowly instilled into the lungs via the trachea cannula and the fluid immediately aspirated. This process was repeated three times. This procedure resulted in 40–50% recovery of BAL fluid from the lungs of each guinea pig. Total cell counts in each BAL sample were determined by

counting. To differentiate the different cell types in the BAL, cytospin preparations were made and the resulting slides were fixed and stained for differential cell analysis. On each slide, in an area selected at random, 200 cells were counted under light microscope, with cells being classified as neutrophils, eosinophils and mononuclear cells according to standard morphologic criteria.

Acknowledgments

We thank Grace Wells, William Hageman, Jeffery Hall, Bryan Raferty, Wensheng Lang, John Masucci, and Gary Caldwell for pharmacokinetic and pharmacodynamic studies.

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