

Short Communication

Improvement of Pulmonary Function by Oral Treatment with a VLA-4 Antagonist in a Mouse Asthmatic Model

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Received August 31, 2012; Accepted December 19, 2012

Abstract. We investigated in vivo efficacies of the newly synthesized VLA-4 antagonist Compound A {*trans*-4-[1-[[2,5-Dichloro-4-(1-methyl-3-indolylcarboxamido)phenyl]acetyl]-(4*S*)-methoxy-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid} on *Ascaris* antigen-induced airway inflammation and hyperresponsiveness in a murine asthmatic model. Oral administration of Compound A significantly inhibited eosinophil infiltration into BALF and airway hyperresponsiveness 48 h after the antigen challenge. Histologic analysis of the lung sections confirmed the BALF result and revealed suppression of edema and mucus hyperplasia at 8 and 48 h after the challenge, respectively. These findings clearly show that orally active Compound A has therapeutic potential for treatment of asthma.

Keywords: VLA-4, eosinophil, airway hyperresponsiveness

Asthma is a chronic inflammatory disease characterized by antigen-induced airway hyperresponsiveness (AHR), leukocytes accumulation into inflamed sites, airway mucosal hyperplasia, and airway wall remodeling. Airway response to antigen can be classified into two phases: immediate phase airway response (IAR) and late phase airway response (LAR), and LAR is recognized as an important pathological step for progression of asthma. LAR is characterized by excessive inflammation of the airway accompanied with influx of eosinophils, T cells, and other inflammatory cells into inflamed pulmonary tissues. Migration of inflammatory cells into the inflamed sites requires the interaction between integrin on leukocytes and cell adhesion molecules on pulmonary endothelial cells (1). Among the adhesion molecules, very late antigen-4 (VLA-4), composed of α_4 and β_1 subunits, is recognized as a prime target for inflammatory disease, for it is required for interaction between activated endothelial cells and inflammatory cells such as eosinophils, T cells, basophils, and mast cells, but not neutrophils. Indeed, treatment with VLA-4 antagonists and anti- α_4 integrin antibody has been reported to be effective in

animal models (2 – 8).

trans-4-[1-[[2,5-Dichloro-4-(1-methyl-3-indolylcarboxamido)phenyl]acetyl]-(4*S*)-methoxy-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid (referred as Compound A hereafter), has been identified as a highly active VLA-4 antagonist with good bioavailability in rats, dogs, and monkeys (8). This compound is a powerful VLA-4 antagonist with an IC₅₀ value of 5.4 nM in the VCAM-1/VLA-4 binding assay (8). Additionally, the compound is highly selective for $\alpha_4\beta_1$ by more than 1000-fold compared with other integrins, such as LFA-1, $\alpha_4\beta_7$, and GPIIb/III3 (8). In this study, we report the in vivo efficacies of Compound A by oral administration in an antigen-induced murine asthmatic model and the efficacies are confirmed by further histological examination.

We used 7-week old female BALB/c mice to generate the murine asthmatic model by oral administration of cyclophosphamide (150 mg/kg) (Day 0), followed by intraperitoneal injection of *Ascaris suum* extract (500 μ g) emulsified in 4.5 mg aluminum hydroxide on Day 2 and Day 14 for systemic immunization. Eight days after the second immunization (Day 22), the animals were intratracheally challenged with 30 μ L saline or the *Ascaris* antigen (300 μ g) under anesthesia. Vehicle (0.5% methyl cellulose) or Compound A was orally administered at 8, 24, and 32 h after the challenge, in addition to 15 min

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Published online in J-STAGE
doi: 10.1254/jphs.12198SC

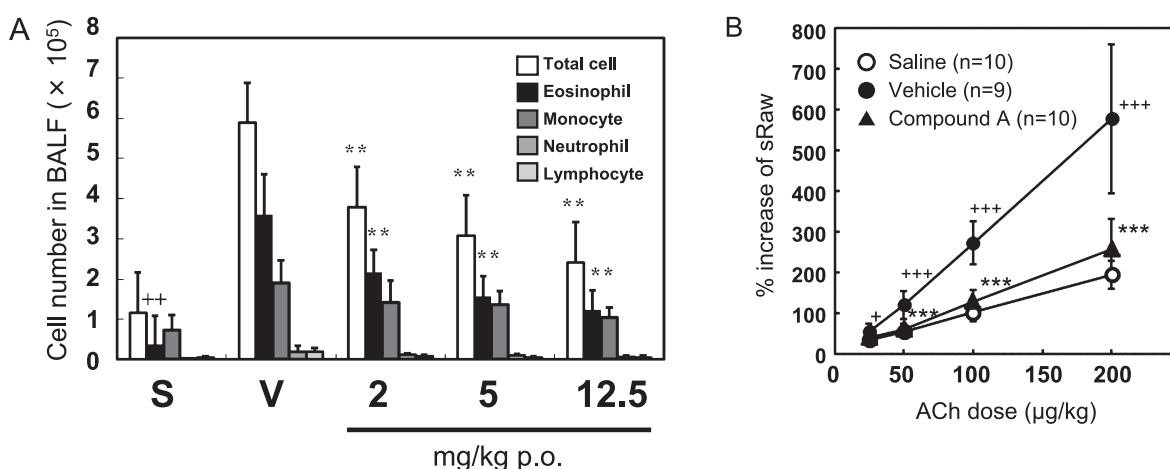


Fig. 1. In vivo effect of Compound A on airway inflammation and AHR to Ach in *Ascaris suum*-induced murine asthmatic model. A) Numbers of eosinophils, monocytes, neutrophils, and lymphocytes in saline-challenged (S) or antigen-challenged plus vehicle (V) or Compound A (2, 5, or 12.5 mg/kg)-treated mice are shown. Cell numbers are represented as the mean $\pm$ S.E.M. Eight mice were tested per group. $^{++}P < 0.01$, compared to V (Student's *t*-test); $^{**}P < 0.01$, compared to V (Dunnett's test). B) Airway response to Ach (25, 50, 100, and 200 μ g/kg) was measured at 48 h after the antigen challenge. Nine or ten mice per group were treated with Compound A (12.5 mg/kg) or vehicle 15 min before and 8, 24, and 32 h after the antigen challenge. Percent increase of Raw was calculated as the ratio of the peak of Raw to baseline. $^{*}P < 0.05$ and $^{+++}P < 0.001$, compared to Saline (Student's or Aspin-Welch's *t*-test); $^{***}P < 0.001$, compared to Vehicle (Student's or Aspin-Welch's *t*-test).

prior to the challenge.

The anti-inflammatory effect of Compound A was determined by measuring the number of infiltrated leukocytes in the bronchoalveolar lavage fluid (BALF). At 48 h after the challenge, the animals were sacrificed and the BALFs were obtained. The BALF cells were counted using a Sysmex CDA-500 cell counter and centrifuged onto cytospin slides (Cytospin2; Shandon, Cheshire, UK). The preparations were stained with Wright's stain to obtain differential leukocyte counts.

The AHR to acetylcholine (ACh) was measured as follows: Mice were anesthetized and the trachea was cannulated and connected to a rodent ventilator (Mini vent type 845; Harvard Apparatus GmbH, March-Hugestetten, Germany) with an in-line pressure transducer (TRD 4510; Buxco, Sharon, CT, USA), coupled to a pulmonary mechanics analyzer (Bio System XA; Buxco, Troy, NY, USA). Flows were determined by measuring the differential pressure across eight layers of 400-mm mesh wire cloth covering a 1.3-cm hole in a plethysmograph box (Plyan-M; Buxco). After establishing a stable baseline of specific airway resistance (sRaw), ACh was cumulatively administered (25 – 200 μ g/kg) via the tail vein, and changes in sRaw were monitored.

For histological examination, the lungs were resected at 8 and 48 h after the challenge and fixed in 10% phosphate-buffered formalin; the HE-stained sections were used for assessment of general morphology and extravascular infiltration of cells. Alcian Blue-stained

samples were used to depict excreted mucus and mucus-containing goblet cells. The development of each asthma symptom in the lung sections were subjectively semi-quantified by the following scores: 0, absent; 1, slight; 2, moderate; 3, severe.

Plasma concentrations of Compound A (5 mg/kg) were determined by an LC/MS/MS. The antigen-immunized mice were orally treated with the compound and challenged intratracheally. Blood was collected under anesthesia at 1, 2, and 4 h after the challenge. A 50- μ L sample of plasma and 160 μ L of 100% methanol containing the internal standard (Warfarin, 100 ng/mL) were mixed thoroughly and centrifuged. A 10- μ L aliquot of the obtained supernatant was injected onto the LC/MS/MS system (LC: Alliance 2795, MS: TSQ7000). Results were expressed as the peak area ratio of Compound A to the internal standard obtained from LC/MS/MS analyses.

Compound A, *trans*-4-[1-[[2,5-Dichloro-4-(1-methyl-3-indolylcarboxamido)phenyl]acetyl]-(4*S*)-methoxy-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid, was identified and synthesized at Daiichi Sankyo Co., Ltd. (8) as a novel, specific antagonist of VLA-4. All data were given as the mean $\pm$ S.E.M. *P*-values were obtained by statistical analysis, as noted in the figure legends. All the animal experiments were conducted with the approval of the Animal Experiment Ethics Committee of Daiichi Sankyo Co., Ltd.

From the aspect of the mode of action, Compound A

Table 1. Grading of cell infiltration, edema, and mucus secretion in each lung section

Groups	8 h after challenge										48 h after challenge										
	As/Vehicle n = 5				As/CpdA n = 5				As/Vehicle n = 5				As/CpdA n = 5				S/Vehicle n = 5				
	Grade	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Inflammatory cell infiltration, peri-bronchiolar/vascular and septal		0	3	2	0	1	4	0	0	0	0	0	5	0	4	1	**0	5	0	0	**0
Edema, perivascular		0	1	4	0	5	0	0	*0	4	1	0	0	2	3	0	0	5	0	0	0
Increased mucus secretion / goblet cell metaplasia in bronchiolar epithelium		2	1	0	2	2	2	0	1	0	1	1	3	0	2	3	0	2	2	1	*0

The severity of lung pathology was graded semi-quantitatively on a scale ranging from 0 to 3 for the following features: 1) cell infiltration: 0 = no or occasional cells, 1 = few loosely arranged cells, 2 = many cells in the peripheral parts of the perivascular space, 3 = numerous cells in the perivascular space; 2) edema: 0 = no visible edema, 1 = less than 10% of the parenchyma, 2 = 10% – 50% of the parenchyma, 3 = more than 50% of the parenchyma; 3) mucus secretion: 0 = no obstruction, 1 = one airway partially or totally obstructed, 2 = two airways partially or totally obstructed, 3 = three or more airways partially or totally obstructed. Each group consisted of 5 mice. Data were analyzed by the Wilcoxon rank-sum test. **P* < 0.05, ***P* < 0.01, compared with As/Vehicle.

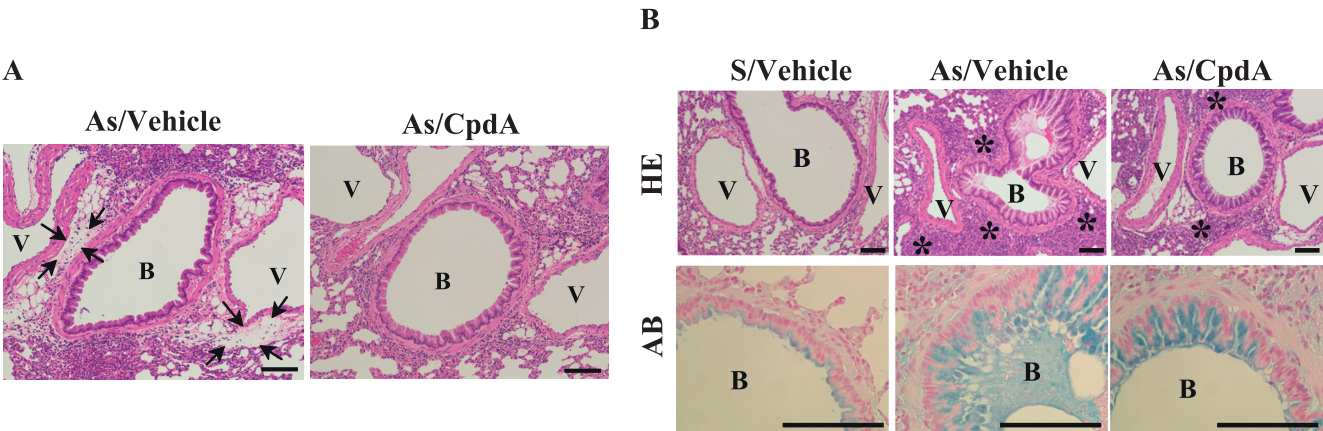


Fig. 2. Effect of Compound A on edema formation, inflammatory cell infiltration, and mucus secretion in lung tissues. Lung sections were analyzed from mice, challenged with saline (S/Vehicle) or *Ascaris suum*, with 0.5% methylcellulose (As/Vehicle) or Compound A (As/CpdA) administration. Mice were treated with 0.5% methylcellulose or Compound A (5 mg/kg) at 15 min before and 8, 24, and 32 h after the challenge and sacrificed at 8 or 48 h after the challenge. A) The arrows denote the presence of edema around the vessel (V) and the bronchi (B), following antigen challenge. B) Migrated cells are indicated by stars. As/Vehicle showed more severe peribronchiolar and perivascular inflammatory cell infiltration after Hematoxylin-Eosin (HE) staining, compared with As/Cpd. Goblet cell metaplasia and mucus plugs after Alcian blue (AB) staining were reduced in As/Cpd. Bar = 100 μ m.

is expected to control LAR by predominantly blocking infiltrating inflammatory cells into inflamed tissues. In the *Ascaris*-induced asthmatic model, we observed a significant increase in the number of total leukocytes and eosinophils into BALF in vehicle-treated mice. Oral administration of Compound A significantly reduced the infiltration of total leukocytes and eosinophils in a dose-dependent manner (Fig. 1A). The compound induced a 45%, 63%, and 73% decrease in eosinophil efflux into BALF, at doses of 2, 5, and 12.5 mg/kg, respectively. In this model, the eosinophils accounted for 20% and 61% of the total leukocytes in the saline-

challenged (group S in Fig. 1) and antigen-challenged vehicle-treated mice (group V in Fig. 1), respectively, indicating induction of eosinophilia, which found to be the main target of the inhibitory effect of Compound A. We next examined the effect of Compound A on pulmonary function. In vehicle-treated mice, AHR was observed after intravenous ACh challenge, with significant increase in sRaw, compared with the saline-challenged mice (Fig. 1B). Compound A at 12.5 mg/kg showed a dramatic reduction of AHR with ACh almost to the basal level, suggesting that Compound A has therapeutic potential for asthma treatment by oral

administration.

The efficacies of Compound A were further confirmed by histological examination and summarized in Table 1. Asthmatic symptoms such as mucus secretion and inflammatory cell infiltration into lungs were inhibited as well as edema, which is supposed to be predominantly mediated by degranulation of mast cells in the early allergic response. As shown in Fig. 2A, the development of lung edema in the perivascular region was observed in vehicle-treated mice at 8 h after the antigen challenge, but not in Compound A-treated mice. Effect of VLA-4 inhibitors on mast cells is controversial, and some inhibitors were reported to suppress mast cell function in animal asthma models (9–12). Although the mechanism for how Compound A suppresses mast cell activation is not clarified, this potent VLA-4 inhibitor also inhibits IAR caused by mast cells in either a direct or indirect manner. Inhibition of edema at 48 h by Compound A was slightly weak compared with the inhibition at 8 h (Table 1). This might be due to insufficient plasma concentration of Compound A required for maintaining suppression of degranulation, but the precise reasons remain unclear. After edema receded at 48 h after the challenge, severe inflammatory cell infiltration (largely eosinophils and monocytes) in the perivascular and peribronchiolar regions was observed in the antigen-challenged mice. Significant blockade of cell infiltration was observed in histological analysis, confirming the BALF result (Fig. 2B). Moreover, Alcian blue staining revealed that the treatment with Compound A partially suppressed mucus secretion by epithelial cells and hypermetaplasia of the goblet cells (Fig. 2B). The BALF result shows Compound A at 5 mg/kg exerts potent inhibition of eosinophil infiltration; however, in the table, infiltrated cells including monocytes in the lung interstitial area would contribute to edema and mucus secretion, to some extent. It has been reported that allergen-induced inflammatory cell persistence results in prolonged goblet cell metaplasia and mucus secretion, which is considered as a major contributor to airway obstruction (13). Taken together, these findings indicate that Compound A would improve pulmonary function.

In order to examine the pharmacological potential of Compound A, we further analyzed its pharmacokinetics. In the animal model described above, the plasma concentration of Compound A rose from 109 ng/mL (150 nM) at 1 h after administration to 193 ng/mL (265 nM) at 2 h, and then decreased to 37 ng/mL (51 nM) at 4 h. These concentrations exceed the *in vitro* IC₅₀ value for VLA-4/VCAM-1 by more than 10-fold. Thus, it is conceivable that multiple treatment of Compound A (bid for 2 days) might maintain its plasma concentration enough to selectively act on VLA-4-expressing inflam-

matory cells.

In conclusion, we showed that Compound A, a specific and orally active VLA-4 antagonist, drastically reduced asthmatic symptoms such as edema, eosinophil infiltration, goblet cell hyperplasia, mucus secretion, and AHR. Thus, this compound has significant therapeutic potential as an anti-asthma agent.

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