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Original Article

Effects of TMK-688, a potent 5-lipoxygenase inhibitor, on dual-phase asthmatic response in conscious guinea pigs sensitized with ovalbumin

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Abstract

We evaluated the anti-asthmatic effects and mechanism of action of TMK-688, a potent 5-lipoxygenase inhibitor, on the dual-phase asthmatic response and on airway inflammation in conscious guinea pigs sensitized with ovalbumin (OA). TMK-688 inhibited both the immediate and the late asthmatic response (LAR) after administration of a single oral dose of 3.2 or 10 mg/kg 2 h before OA challenge. Pretreatment with TMK-688 also inhibited airway hyperresponsiveness to acetylcholine. The increase in eosinophils in bronchoalveolar lavage fluid and the production of reactive oxygen, an index of cell activation during LAR, was also suppressed by TMK-688. These findings suggest the following inhibitory mechanism of LAR by TMK-688: (i) a reduction of eosinophil accumulation in airways; (ii) the inhibition of the immediate asthmatic response; (iii) the inhibition of airway hyperresponsiveness; and (iv) the suppression of the generation of reactive oxygen from bronchoalveolar lavage cells.

Key words: airway hyperreactivity, bronchoalveolar lavage cell, dual-phase asthmatic response model, guinea pigs, late asthmatic response, reactive oxygen, TMK-688.

Introduction

TMK-688 was developed as a potent 5-lipoxygenase inhibitor with histamine antagonism.[1] When administered orally, TMK-688 reduces leukotrienes B4 and C4 in both nasal-lavage fluids and in the lungs of allergen-challenged guinea pigs.[2] TMK-688 also shows anti-anaphylactic activity on the passive cutaneous anaphylaxis (PCA) test in the rat and on the Forssman and Arthus reactions in the guinea pig.[3]

TMK-688 was developed as an anti-allergy agent. At an oral dose of 20 mg/day TMK-688 has been shown, in clinical trials in Japan, to be safe and effective in adults with bronchial asthma.

In the present study we examined the effects of TMK-688 on the dual-phase asthmatic response, including the immediate (IAR) and late asthmatic response (LAR), cell accumulation in bronchoalveolar lavage (BAL) fluid in ovalbumin (OA)-sensitized guinea pigs and non-specific airway hyperresponsiveness after acetylcholine (ACh) challenge in guinea pigs.

Methods

Animals and preparation of the dual-phase asthmatic model

Male Hartley guinea pigs weighing 250–300 g were purchased from Japan SLC (Shizuoka, Japan) and were used to prepare the dual-phase asthmatic model with Tohda's method.[4] Guinea pigs were housed in a humidity and temperature-controlled room (12 h:12 h light:dark cycle) with free access to food and water.

Andersson's method[5] was modified as follows. Guinea pigs received intraperitoneal injections of 30 mg/kg cyclophosphamide (Shionogi Pharmaceutical Co. Ltd, Osaka, Japan). Two days later, 1 mg/0.5 mL OA (grade V; Sigma Chemical Co., St Louis, MO, USA) containing 100 mg aluminum hydroxide as an adjuvant (Wako Pure Chemical Industries, Osaka, Japan) was injected intraperitoneally. Three weeks later, 10 μg/0.5 mL OA

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containing 100 mg aluminum hydroxide was injected intraperitoneally for sensitization. Airway responsiveness after OA challenge was measured 3 weeks later.

Administration of compound

TMK-688 was dissolved in Tween 80 (NIKKOL TO–10 M; Nippon Chemical, Tokyo, Japan) and was raised to a sufficient volume with double-distilled water. The final concentration of Tween 80 was 5%. Then, 3.2 or 10 mg/kg TMK-688 was administered orally with a probe (Magen sonde; Natsume Seisakusyo Co. Ltd, Tokyo, Japan) to guinea pigs 2 h before OA or ACh challenge. Control guinea pigs received 4 mL/kg of the appropriate vehicle.

Measurement of specific airway resistance

Specific airway resistance was measured with Pennoch's method[6] as follows. The guinea pig was placed inside a two-chambered body plethysmograph (PYLUN/2P: Chamber Model-P; Buxco Electronics Inc., Sharon, CT, USA). Box pressure was measured with a pressure transducer (DP-45; Buxco). Specific airway resistance (SAR; cmH_2O/s) was calculated from the box flow signal with a non-invasive respiratory analyzer (PUMR + SAR; Buxco).

After baseline measurement of SAR, guinea pigs were challenged for 1 min with OA aerosol (1 mg OA/kg) administered with a nebulizer (DeVilbiss 646; Shizume Medical, Tokyo, Japan) directed into a face mask placed on the guinea pig. Specific airway resistance was measured at 1, 3 and 5 min and every hour for 7 h after challenge.

Airway hyperresponsiveness test with ACh challenge

Airway hyperresponsiveness was measured with the same method used for SAR measurement. Saline or increasing concentrations of ACh (4.9, 9.8, 20, 39 and 78 μg/mL in saline) were aerosolized (8.2 L/min) for 30 s at 3 min intervals with a nebulizer. The airway hyperresponsiveness was measured before and after challenge (1, 3 and 5 min and every hour for 7 h after challenge). The airway hyperresponsiveness was calculated from the box flow signal as described by Pennoch.[6]

Measurement of cell content and cell construction in BAL fluid after OA challenge

Guinea pigs given vehicle or TMK-688 were divided into three groups, as described earlier, to determine the changes in cell content of BAL fluid before and 5 min and 4 h after OA challenge. Guinea pigs were anesthetized with 50 mg/kg sodium pentobarbital, i.p. The trachea was cannulated with a probe at the carina. The lungs were lavaged four times with saline (5 mL/kg) through the probe. Seventy to 80% of the lavage fluid was consistently recovered. An aliquot of the lavage fluid was centrifuged (200 g for 5 min at 4°C) to pellet cells. The pellet was resuspended in saline and the total number of cells was counted with a standard hemocytometer after staining with 0.2% trypan blue solution (Wako). The remainder of the lavage fluid was centrifuged at 200 g for 5 min for the differential cell counts. The pellet was stained with May-Gruenwald Giemsa (Wako) and each cell was counted.

Measurement of reactive oxygen generation from BAL cells

Cell preparation

Bronchoalveolar lavage cells were obtained from OA-sensitized guinea pigs with the method described earlier and were resuspended with Hank's balanced salt solution (HBSS; Whittaker Bioproducts, Walkersville, MD, USA) at 1×10^6 cells/mL.

Sample preparation

Control solution Dimethylsulfoxide (Merck, Darmstadt, Germany) was diluted with HBSS to a concentration of 0.5%.

TMK-688 solution TMK-688 was dissolved in dimethylsulfoxide and was diluted with HBSS to 0.1, 0.5 and 1.0 μg/mL.

Measurement of reactive oxygen generation from BAL cells

The cell suspension (80 μL) was incubated with 20 mL TMK-688 solution or control solution for 10 min at 37°C, followed by the addition of 50 μL luminol solution (2×10^{-4} mol/L; Futaba Medical, Tokyo, Japan). Luminol-

dependent chemiluminescence (LDCL) was measured with a lumiphotometer (TD-4000; Futaba Medical) after stimulation with 50 μL calcium ionophore A23187 (25 μg/mL; Sigma Chemical Co.). The integrated value for 600 s at 37°C was recorded.

Ethical standards

All experiments were performed according to institutional guidelines for the care and use of laboratory animals.

Statistical analysis

Data are expressed as the mean±SEM and were compared for statistically significant differences with the Student's *t*-test or Dunnett's statistics. Differences with a *P* value less than 0.05 were considered significant.

RESULTS

Airway response after OA challenge in guinea pigs

In each of the TMK-688-treated groups (3.2 or 10 mg/kg) and in the untreated group, 10 guinea pigs were challenged with OA and changes in airway resistance were recorded. The IAR were observed 1–5 min after challenge and LAR were observed 4–7 h after challenge. In each animal, the difference in airway resistance before and after challenge was calculated to determine the increase in airway resistance. The mean integrated value 1–5 min after challenge (IAR) and that after 4–7 h (LAR) are shown in Table 1.

The increase in airway resistance with IAR was 26.03±0.81 cmH_2O/S in the untreated group. However, the increase was slightly less in guinea pigs receiving 3.2 mg/kg TMK-688 (19.59 ± 3.69) and was significantly less in guinea pigs receiving 10 mg/kg TMK-688 (10.87 ± 3.05).

The increase in airway resistance with LAR was 6.85 ± 0.88 in the untreated group. However, the increases were significantly less in groups treated with TMK-688 at both 3.2 and 10 mg/kg (2.91±1.13 and 1.16 ± 0.56, respectively).

Table 1. Integrated value of change in specific airway resistance before and after challenge at the immediate and late asthmatic response in guinea pigs treated with TMK-688

Treatment	*n*	Integrated value of △SAR	
		IAR	LAR
None	10	26.03±0.81	6.85±0.88
TMK-688			
3.2 mg/kg	10	19.59±3.59	2.91±1.13*
10 mg/kg	10	10.87±3.05*	1.16±0.56*

The occurrence of the immediate asthmatic response (IAR) and late asthmatic response (LAR) in ovalbumin-challenged guinea pigs. Guinea pigs were given either vehicle or TMK-688 orally 2 h before challenge. Definitions of IAR and LAR are given in the Methods.

Results are expressed as the mean ± SEM. Differences between guinea pigs not treated and treated with TMK-688 were assessed with analysis of variance using Dunnett's statistics. *$P<0.05$ versus none.

Airway hyperresponsiveness after ACh challenge in guinea pigs

Figure 1 shows the effect of TMK-688 (3.2 mg/kg) on SAR after ACh challenge. The SAR in unsensitized guinea pigs was not increased by challenge with ACh at doses up to 78 μg/mL. The increase in SAR was completely blocked by TMK-688.

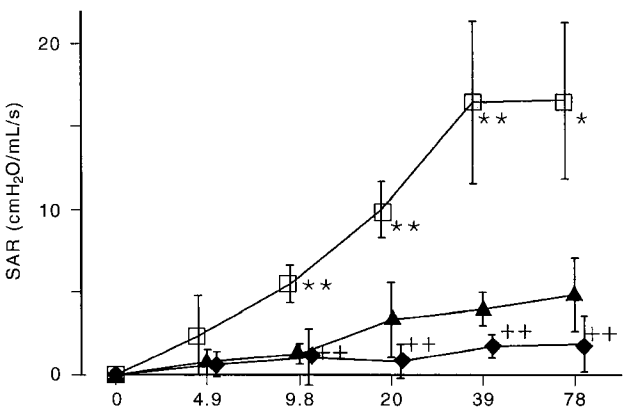

Fig. 1 Effect of TMK-688 on specific airway resistance (SAR) in guinea pigs challenged with acetylcholine (ACh). Guinea pigs were sensitized with ovalbumin before ACh challenge and received either vehicle or 3.2 mg/kg TMK-688 2 h before the first ACh challenge. Saline or increasing concentrations of ACh were nebulized for 30 s at 3 min intervals. Results are expressed as the mean±SD. The significance of differences between the two groups was assessed with Student's *t*-test. (*$P<0.05$; **$P<0.01$ *vs* unsensitized; ††$P<0.01$ *vs* control.) (▲), unsensitized ($n=3$); (□), control ($n=4$); (◆), TMK-688 ($n=3$).

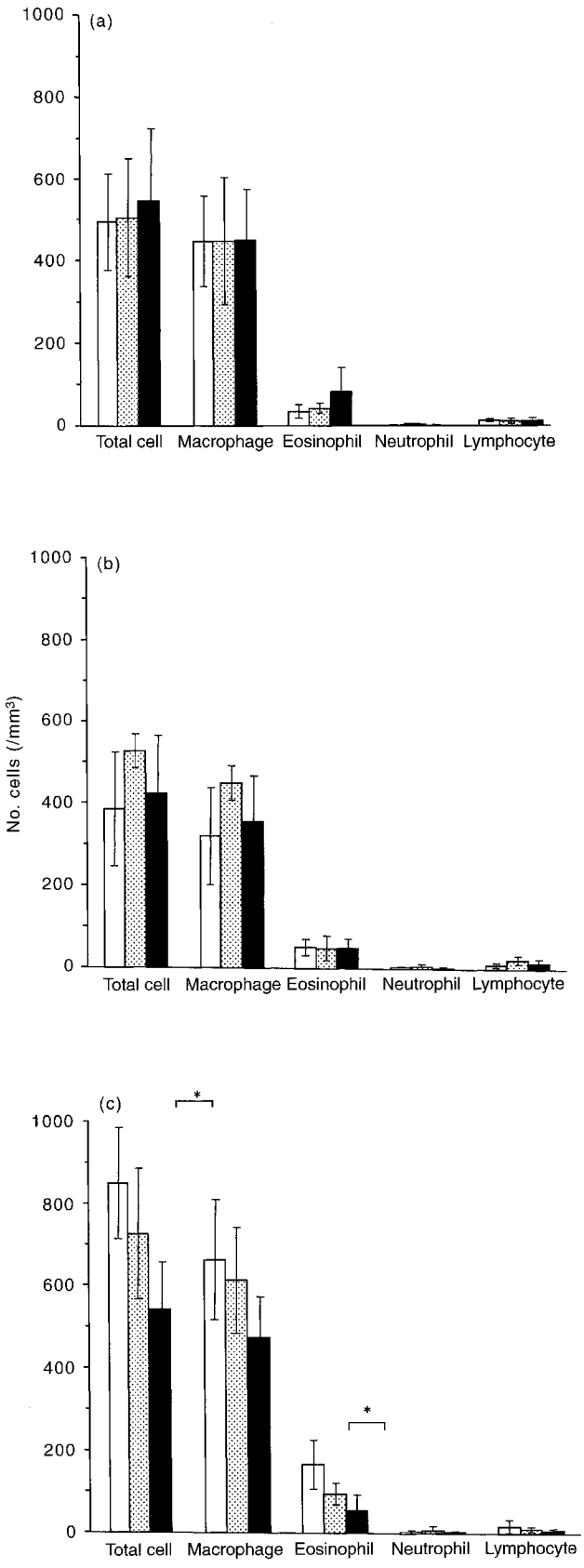

Fig. 2 Cell content and construction in bronchoalveolar lavage fluid from guinea pigs given either vehicle or TMK-688 (3.2 or 10 mg/kg) orally 2 h before ovalbumin (OA) challenge. Bronchoalveolar lavage was performed (a) before, (b) immediately after or (c) 4 h after OA challenge. Results are expressed as the mean±SD for at least five guinea pigs per group. The significance of differences among groups was assessed with analysis of variance using Dunnett's statistics (*$P<0.05$). (□) sensitization+OA challenge; (⊡), sensitization+TMK-688 (3.2 mg/kg, p.o.)+OA challenge; (■), sensitization+TMK-688 (10 mg/kg, p.o.)+OA challenge.

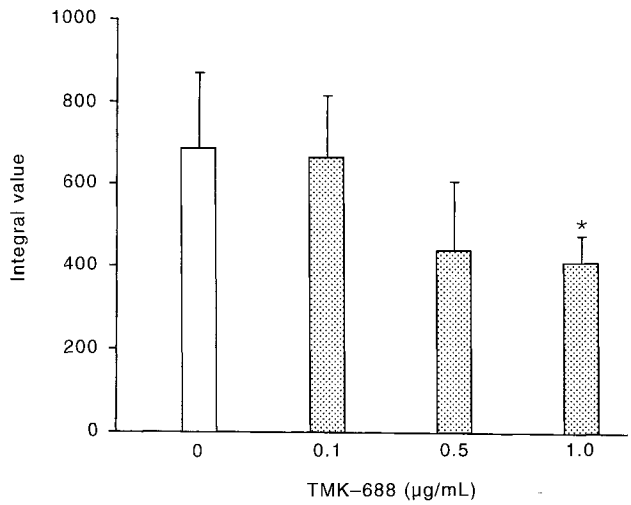

Fig. 3 Effects of TMK-688 (⊡) on reactive oxygen generation from bronchoalveolar lavage cells. Bronchoalveolar lavage cells were incubated with TMK-688 (0.1, 0.5 and 1.0 μg/mL) for 10 min at 37°C followed by stimulation with calcium ionophore (A23187). As a positive control (□), bronchoalveolar lavage cells were incubated without TMK-688. Figure 3 represents integrated values of luminol-dependent chemiluminescence. Results are expressed as the mean±SD. The significance of differences among groups was assessed with analysis of variance using Dunnett's statistics (*$P<0.05$).

Cell content in BAL fluid

In BAL fluid obtained before OA challenge, the total numbers of cells and the numbers of cells of all types did not differ between the control group and the TMK-688-treated groups (Fig. 2a).

In BAL fluid obtained 5 min after OA challenge (IAR) the numbers of cells of each type did not differ significantly between the three groups (Fig. 2b). In BAL fluid obtained 4 h after OA challenge (LAR), the numbers of total cells, macrophages, and eosinophils in control

animals were higher than before challenge or at 5 min (Fig. 2c).

However, in animals that received TMK-688 2 h before challenge, the increase in the numbers of total cells, macrophages and eosinophils were inhibited dose dependently by TMK-688. At a dose of 10 mg/kg TMK-688, the numbers of total cells and eosinophils were significantly lower.

Reactive oxygen-generating activity of BAL cells

TMK-688 at a dose of 1.0 μg/mL significantly suppressed the generation of reactive oxygen from BAL cells (Fig. 3).

Discussion

The LAR can occur after IAR in some asthmatic subjects challenged with an antigen[7] and is suspected to be closely related to intractable asthma for several reasons. First, the airway during LAR is hyperresponsive to stimulation.[8,9] Second, conventional short-acting β_2-adrenoceptor agonists do not inhibit either LAR or the accompanying increase in non-specific airway responsiveness.[10,11] Third, elevated levels of circulating blood eosinophils and bronchoalveolar eosinophilia are associated with LAR.[12,13] Fourth, the tunica mucosa tracheae is damaged in patients with LAR. Although IAR is thought to occur because of IgE-mediated events involving cell degranulation and mediator release, the mechanism governing LAR is still poorly understood. In sensitized subjects, LAR can be provoked with various allergens, including simple chemicals that are difficult to prove to be allergens, and is induced by exercise even in asthmatic patients.[14] Although we cannot exclude the involvement of a non-immunologic mechanism in LAR, the association of airway inflammation with LAR is suspected.[12]

Some investigators have reported that LAR is induced by inflammation after bacterial or viral challenge in animal models. Recently, inflammation due to allergy, especially that involving neutrophils, basophils, lymphocytes, monocytes and alveolar macrophages, is attracting attention.

Leukotriene is thought to play a major role as a chemical mediator in LAR. TMK-688 has been confirmed *in vivo* to inhibit leukotriene synthesis by inhibiting 5-lipoxygenase.[2] Because its profile suggests that it may be effective as an anti-asthmatic, we have evaluated TMK-688 in animals and in human subjects. In LAR in OA-sensitized guinea pigs, oral administration of 1–10 mg/kg TMK-688 inhibits increases in leukotriene B4 and C4 in lung tissue.[2] In asthmatic patients, oral administration of 20 mg/day TMK-688 was useful (T Miyamoto *et al.*, unpubl. obs., 1991). The doses used in these human and animal studies were consistent with an *in vivo* blood concentration sufficient to inhibit 5-lipoxygenase activity.[1] These findings suggest that inhibition of leukotriene synthesis is involved in the anti-asthmatic action of TMK-688.

In the present study we counted the numbers of cells of different types in BAL fluid to investigate the mechanism of action of TMK-688 on LAR. At an oral dose of 3.2 mg/kg, TMK-688 inhibited LAR and modulated the recruitment of eosinophils and neutrophils in BAL fluid. A close relationship between LAR and the influx of inflammatory cells into the bronchial lumen has been shown in some animal models.[16–27] Therefore, TMK-688 may prevent LAR by inhibiting the recruitment of inflammatory cells.

We found that TMK-688 both inhibited the increase in SAR during IAR and inhibited the LAR. Robertson *et al.*[28] attribute the LAR of the dual-phase asthmatic response to a series of IgE-mediated events. The effects of TMK-688 on SAR may result from the prevention of IAR, which may then inhibit the LAR. The effect of TMK-688 on LAR may result from the inhibition of immunologic mediators because TMK-688 is long-lasting and inhibits both the generation of leukotrienes *in vivo* and airway contraction induced by histamine challenge. In some histologic studies, airway inflammation was increased even in patients with mild asthma.[29,30] Ozone inhalation or viral infection cause inflammation and hyperreactivity in the airway.[31–35] Airway inflammation is thought to play an important role in the development of bronchial asthma and in the increase in airway hyperresponsiveness. Reactive oxygen is thought to play an important role in airway inflammation. By generating reactive oxygen in response to various stimulants, such as leukotriene B4[36] and platelet-aggregating factor,[37] eosinophils induce airway damage.[38] Uenishi[39] reported that the LDCL of BAL cells is increased in OA-challenged guinea pigs, that the peak value of LDCL correlates with the amount of antigen, that BAL cells are activated by OA-challenge and that they participate in the development of airway inflammation through the activation of eosinophils. He also reported that these factors were the cause of LAR.[39]

In the present study TMK-688 inhibited the LDCL of BAL cells at a final concentration of 1.0 μg/mL. This finding suggests that TMK-688 is related to the inhibition of LAR through the inhibition of inflammatory cell activation.

Asthmatic patients are hyperresponsive to bronchoconstrictors, such as histamine, methacholine and ACh. We investigated the effect of TMK-688 on airway hyperresponsiveness in ACh-challenged guinea pigs. TMK-688 inhibited airway hyperresponsiveness at an oral dose of 3.2 mg/kg. Airway inflammation plays an important role in increasing airway hyperresponsiveness. Histologic studies have revealed the presence of airway inflammation even in patients with mild asthma.[40,41] Activated inflammatory cells generate reactive oxygen species, such as superoxide[42] and hydroperoxide,[42] which are important in the inflammatory process. As BAL macrophages of asthmatic subjects generate more reactive oxygen than do those of normal subjects,[43,44] reactive oxygen may play a critical role in airway hyperresponsiveness. In the present study, TMK-688 inhibited reactive oxygen generation and airway inflammation and then inhibited airway hyperresponsiveness to ACh. The ineffectiveness of TMK-688 against baseline lung resistance suggests that the inhibition of airway hyperresponsiveness was not due to bronchodilation.

The LAR in this model accurately reflects the pathology of intractable asthma. TMK-688 inhibits the increase of airway hyperresponsiveness and prevents the LAR. These findings suggest the usefulness of TMK-688 as an anti-asthmatic drug.

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