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# Anti-inflammatory and bronchodilator properties of KF19514, a phosphodiesterase 4 and 1 inhibitor

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**Abstract**

We investigated the effects of KF19514 (5-phenyl-3-(3-pyridyl)methyl-3H-imidazo[4,5-*c*][1,8]naphthyridin-4(5H)-one) on bronchoconstriction and allergic inflammation in guinea pigs and on tumor necrosis factor-α production in mice. KF19514 inhibited phosphodiesterase 4 ($IC_{50}$ = 0.40 μM) and phosphodiesterase 1 ($IC_{50}$ = 0.27 μM) derived from canine tracheal smooth muscles. KF19514 relaxed contracted tracheal smooth muscle and had a potent inhibitory effect on antigen-induced bronchoconstriction ($EC_{50}$ = 0.058 μM) in vitro. Intravenous administration of KF19514 inhibited histamine-induced bronchoconstriction ($ID_{50}$ = 2.8 μg/kg i.v.). Moreover, oral administration of KF19514 inhibited anaphylactic bronchoconstriction ($ID_{50}$ = 0.2 mg/kg p.o.), and eosinophil infiltration in airway stimulated with platelet-activating factor (PAF) or antigen. KF19514 also produced a significant inhibition of tumor necrosis factor-α production in mice ($ID_{50}$ = 0.023 mg/kg p.o.). Finally, KF19514 completely inhibited antigen-induced hyperreactivity at 0.1 mg/kg p.o. These results demonstrate that KF19514 may have efficacy in the treatment of asthma. © 1997 Elsevier Science B.V.

*Keywords:* Asthma; Eosinophil; Bronchodilator; Hyperreactivity; KF19514; Phosphodiesterase inhibitor

## 1. Introduction

Bronchial asthma is a disease characterized by reversible airway bronchoconstriction. It is now widely accepted that bronchial asthma is characterized by chronic inflammation of the bronchial mucosa, where T cells and eosinophils are prominent (Corrigan and Kay, 1992). Bronchoconstriction is induced by many pharmacological mediators such as histamine, leukotrienes, thromboxanes, neurokinins, acetylcholine, platelet activating factor and so on. Inflammation of the airway in asthma is characterized as infiltration of leukocytes, especially eosinophils. Therefore, the principal therapeutic strategies in the treatment of asthma have been to relax airway smooth muscle and inhibit pulmonary inflammation. In fact, the clinical guideline for the diagnosis and treatment of asthma states that a $\beta_2$-adrenoceptor agonist is used to relax the airway smooth muscle, and steroids to inhibit inflammatory processes (Sheffer, 1992). The elevation of the intracellular concentration of cAMP in both respiratory smooth muscle and inflammatory cells by phosphodiesterase inhibitors might induce bronchodilation and inhibit pulmonary inflammation (De Boer et al., 1992; Cortijo et al., 1993; Underwood et al., 1993; Lagente et al., 1994). At least seven different cyclic nucleotide phosphodiesterase isozymes have now been identified on the basis of their functional characteristics such as substrate specificity and susceptibility to selective inhibitors (Beavo and Reifsnyder, 1990; Nicholson et al., 1991; Beavo et al., 1994). In particular, the potential use of selective phosphodiesterase 4 inhibitors for the treatment of asthma has been extensively studied (Heaslip et al., 1994; Raeburn et al., 1994; Holbrook et al., 1995), based on observations that phosphodiesterase 4 is a principal regulator of cAMP concentrations in respiratory smooth muscles and inflammatory cells. Phosphodiesterase 4 inhibitors such as RP 73401 (Raeburn et al., 1994), CDP 840 (Holbrook et al., 1995) and WAY-PDA-641 (Heaslip et al., 1994), all of which are derivatives of rolipram, have been demonstrated in practice to have an anti-asthmatic effect. The purpose of the present study was to study a

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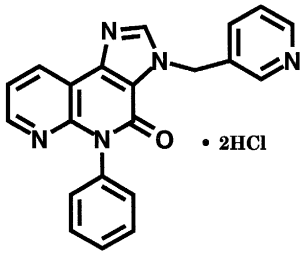


5-Phenyl-3-(3-pyridyl)methyl-3H-imidazo [4,5-c][1,8] naphthyridin-4(5H)-one

***KF19514***

Fig. 1. Chemical structure of KF19514.

novel imidazonaphthyridin derivative, KF19514 (5-phenyl-3-(3-pyridyl)methyl-3H-imidazo[4,5-*c*][1,8]naphthyridin-4-(5H)-one) (Fig. 1) as bronchodilator and with an anti-inflammatory effect in guinea pigs. KF19514 produced functional relaxation of precontracted tracheal smooth muscle strips. Oral administration of KF19514 inhibited eosinophil infiltration in the airway and bronchial hyperreactivity. These results demonstrate that KF19514 is a potent bronchodilator with an anti-inflammatory effect and could have a significant role in the treatment of asthma.

## 2. Materials and methods

### *2.1. Phosphodiesterase*

Slight modifications of the methods of Torphy and Cieslinski (1990) were used to separate the isozymes of phosphodiesterase. Briefly, mongrel dogs (8–15 kg) were killed with an i.v. injection of sodium pentobarbital (65 mg/kg). The tracheae were rapidly removed, frozen in liquid nitrogen and stored at −80°C until use. Tracheal smooth muscles were minced with fine scissors and homogenized with a Polytron® (6 bursts, 20 s/bursts) (Kinematica, Lucerne, Switzerland). The homogenate of the tracheal smooth muscle was centrifuged at $30\,000 \times g$ for 20 min. The resultant supernatant was applied to a diethylaminoethyl (DEAE) cellulose column (DE 52, Whatman, Maidstone, UK). After application of the sample, the column was washed with 70 mM sodium acetate. Phosphodiesterase isozymes were eluted with 70 to 1000 mM sodium acetate as a linear gradient. Fractions were collected and assayed for phosphodiesterase activity.

Phosphodiesterase activity was assayed by the method of Kincaid and Manganiello (1988). Briefly, [$^3$H]cAMP or [$^3$H]cGMP was used as a substrate (1 μM). The reaction was performed in a standard mixture containing 50 mM *N*,*N*-bis(2-hydroxyethyl)-2-amino ethanesulfonic acid (pH 7.2), 1 mM $MgCl_2$ and 0.1 mg/ml soybean trypsin inhibitor. The reaction was initiated with enzyme and incubated at 30°C for 10–30 min depending on the amount of enzyme activity. The reaction was terminated by adding HCl. After complete conversion of the 5′-nucleotide to its corresponding nucleoside by 5′-nucleotidase, the samples were applied to a DEAE-Sephadex A-25 column (Pharmacia, Uppsala, Sweden) and the [$^3$H] nucleoside eluted with water was measured by scintillation counting. The concentration of each drug required to produce 50% inhibition ($IC_{50}$, in μM) was calculated by linear regression analysis of the percent inhibition data.

### *2.2. Preparation for rabbit anti-ovalbumin serum*

Male albino rabbits (2.6–3.0 kg) were sensitized by intramuscular injections of ovalbumin (1 mg/rabbit) emulsified with complete Freund's adjuvant (Yatoron, Tokyo, Japan) followed by three booster injection of ovalbumin (2 mg/rabbit) once weekly for 3 weeks. The serum was obtained 7 days after the last sensitization.

### *2.3. Antigen-, histamine-, or carbachol-induced contraction of trachea strips*

To examine the effects of the test drugs on antigen-induced contraction, male Hartley guinea pigs (350–450 g) were passively sensitized by injection of 1 ml/animal of rabbit anti-ovalbumin serum i.p. and used for experiments 16–24 h later. The animals were killed by $CO_2$ asphyxiation and exsanguination. The tracheae were excised and cleaned of adhering adipose and connective tissues. Tracheal zig-zag strips were prepared by the method of Emmerson and Mackay (1979), followed by equilibration for 1 h in Krebs–Henseleit solution with 95% $O_2$/5% $CO_2$ at 37°C. Ovalbumin was administered at a 10 μg/ml final bath concentration. The contractions were measured with an isotonic transducer (TD-112S, Nihon Kohden Kogyo, Tokyo, Japan) under a constant weight of 1 g, connected to recorders (Type 30066, Yokogawa Hokusin Electric, Tokyo, Japan).

To examine the effects of test drugs on the histamine- or carbachol-induced contraction, tracheal zig-zag strips were prepared from normal guinea pigs. Contractions were induced with histamine (final concentration, $3 \times 10^{-5}$ M) or carbachol ($10^{-7}$ M). The contractions were measured with an isotonic transducer under a constant weight of 0.5 g (histamine) or 5 g (carbachol).

Each concentration produced approximately 80% of the maximum contraction, and each load resulted in about a similar contraction height. Test drugs were administered cumulatively at 7 min intervals after the contraction of tracheal strips had plateaued. Maximum relaxation was induced by adding papaverine ($10^{-4}$ M) at the end of the experiments.

The percent relaxation at each drug concentration was calculated based on the maximum relaxation with papaverine $10^{-4}$ M. The concentration of each drug causing 50% relaxation ($EC_{50}$: M) was calculated by linear regression analysis of the percent relaxation data.

Krebs–Henseleit solution used in this experiment had the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 24.9, D-glucose 11.1.

### 2.4. Histamine-induced bronchoconstriction in vivo

Bronchoconstriction was measured by the modified Konzett and Rössler (1940) method. Male Hartley guinea pigs (350–450 g) were anesthetized with urethane (1.2 g/kg i.p.). The right jugular vein was cannulated for administration of histamine or test drugs and the left carotid artery was used for measurement of blood pressure. The trachea was cannulated and animals were subsequently given gallamine triethiodide (10 mg/kg i.v.) to stop spontaneous respiration. The guinea pigs were then artificially ventilated with a rodent respirator (TB-101, Takashima Shohten, Tokyo, Japan) at 60–70 strokes/min. A side-arm to the tracheal cannula was connected to a bronchospasm transducer (7020, Ugo Basile, Varese, Italy) to measure the air overflow, which was recorded on a polygraph (RM45; Nihon Kohden Kogyo). Histamine (10 μg/kg i.v. as base) was administered at 5 min intervals and transient bronchoconstriction was measured as the increase in air overflow. After stable responses had been obtained, test drugs were administered intravenously 1 min before histamine challenge. The dose of each drug required to produce 50% inhibition ($ID_{50}$, in mg/kg i.v.) was calculated by linear regression analysis of the percent inhibition data.

### 2.5. Anaphylactic bronchoconstriction in vivo

Male Hartley guinea pigs (350–450 g) were passively sensitized with rabbit anti-ovalbumin serum as mentioned above.

Bronchoconstriction was measured as mentioned above. Ovalbumin (5 mg/kg i.v.)-induced bronchoconstriction was measured every minute as a percentage of maximum overflow volume obtained by tracheal clamping at the end of the experiments. The area under the curve to 10 min ($AUC_{0-10}$) of bronchoconstriction was measured using an imaging analyzer (MCID system, Imaging Research, Ontario, Canada). Test drugs were given orally 1 h before antigen challenge. The dose of each drug required to produce 50% inhibition ($ID_{50}$, in mg/kg p.o.) of $AUC_{0-10}$ was calculated.

### 2.6. Platelet-activating factor (PAF) induced eosinophil infiltration in pulmonary airways

The modified method of Sakuma et al. (1991) was used. Guinea pigs in a plastic box ($13 \times 18 \times 25$ cm) were exposed for 10 min to aerosols generated from a PAF solution (1 mg/ml) in bovine serum albumin (0.1%) using an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan). Six hours after PAF exposure, the lungs with trachea were excised after the blood was washed out by injection of physiological saline through the pulmonary artery and 2 ml of 10% buffered formalin was instilled into the lungs to preserve them. For light microscopy, the tracheae and lung tissues were fixed in formalin, embedded in paraffin, and the sections were stained with Hinkelmann's reagent. Eosinophils were specifically stained pink and easily distinguishable from the other cells. All eosinophils infiltrated under the epithelium of trachea and bronchus and around the bronchi with 0.5 mm diameter were counted using a photo microscope (BH-2, Olympus, Tokyo, Japan).

### 2.7. Antigen-induced cellular accumulation and airway hyperresponsiveness

Active sensitization and antigen exposure were performed as previously described by Boichot et al. (1991) with a slight modification. Male Hartley guinea pigs (350–500 g), placed in a plastic box ($30 \times 50 \times 30$ cm) were exposed twice for 30 min to an aerosol of ovalbumin (2 mg/ml) at a 48 h interval for active sensitization. The aerosol was generated with an ultrasonic nebulizer (Ultra-NEB 99, DeVilbiss, Sommerset, PA, USA). Under these conditions, the aerodynamic diameter of the nebulized particles was between 0.5 and 3 μm. 15–20 days after the initial sensitization procedure, the guinea pigs were challenged in the plastic box as described above by exposure to aerosols of five successively increasing concentrations of ovalbumin (0.01, 0.1, 0.5, 1 and 5 mg/ml) for 15 min each. Test drugs were given orally 1 h before the challenge. These increasing concentrations were used to avoid fatal anaphylactic reactions. One hour after the last challenge with the highest concentration of ovalbumin, no apparent sign of respiratory failure was observed in the guinea pigs.

Bronchoalveolar lavages were performed 24 h after antigen-challenge or saline exposure in sensitized guinea pigs. The animals were killed with sodium pentobarbital (100 mg/kg i.p.), then exsanguinated. The trachea was cannulated and 5 ml of warmed (37°C) saline was instilled into the lungs. The lavage fluid was recovered by gentle aspiration and this procedure was repeated twice. The aliquots were pooled and the total volume was measured (recovery was routinely $> 80\%$). The sample was centrifuged ($300 \times g$ at 4°C for 10 min) and the cell pellet was resuspended in saline (1 ml). Total cell counts were determined using a Coulter counter (MEK-440, Nihon Kohden Kogyo) and differential cell counts were done on cytospin slides fixed in methanol and stained with Wright-Giemsa. At least 500 cells were counted according to standard morphological criteria.

To measure airway hyperreactivity, the guinea pigs were anesthetized with urethane (1.2 g/kg, i.p.) and placed in the dorsal recumbent position 24 h after antigen challenge. The trachea was cannulated and the lungs were

mechanically ventilated with a constant volume of 1 ml room air/100 g body weight using a respiratory pump (Ugo Basile) at 60 breaths/min. Spontaneous breathing was abolished by injection of gallamine triethiodide (3 mg/animal) into the jugular vein. After a 10 min stabilization period, five successive 1 min aerosol administrations of acetylcholine hydrochloride (20, 50, 100, 150 and 200 μg/ml) were performed at 5 min intervals with constant monitoring of the bronchopulmonary response. For the inhaled administration of acetylcholine hydrochloride, the aerosol was generated using a DeVilbiss ultrasonic nebulizer connected to the inspiratory line of the respiratory pump with three-way breathing valves. The airflow signal and the pressure signal measured with pressure transducers were electronically integrated using a computerized pulmonary function monitoring system (Model 6, Buxco Electronics, Sharon, CT, USA). Lung resistance ($R_L$) and dynamic lung compliance ($C_{dyn}$) were calculated simultaneously from the airflow and pressure with this system.

### 2.8. Tumor necrosis factor-α (TNF-α) production

TNF-α production induced by lipopolysaccharide in D-galactosamine-sensitized mice was measured by the modified method of Galanos et al. (1979). Briefly, D-galactosamine (500 mg/kg) and lipopolysaccharide (50 μg/kg) were given intravenously to mice, then serum was obtained 1 h after the injection. Test drugs were given orally 30 min before the lipopolysaccharide and D-galactosamine injections. TNF-α content in the mouse serum was determined by enzyme immunosolvent assay using a commercially available TNF-α ELISA kit (Genzyme, Cambridge, MA, USA). $ID_{50}$ values (mg/kg p.o.) were calculated by linear regression analysis of the percent inhibition data.

### 2.9. Protocol approvals

All animals were used in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the experimental protocols were reviewed and approved by the ethical committee of Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo.

### 2.10. Drugs and solutions

KF19514, rolipram and vinpocetine were synthesized by the Chemical Synthesis Division of Pharmaceutical Research Laboratories of Kyowa Hakko Kogyo. The following drugs were used: platelet-activating factor (PAF) C16, C18 mix (Avanti Polar Lipids, Alabaster, AL, USA), histamine dihydrochloride (Wako, Osaka, Japan), carbamic acid ethyl ester (urethane, Tokyo Kasei Kogyo, Tokyo, Japan), theophylline (Nacalai Tesque, Kyoto, Japan), pentobarbital sodium (Nembutal® Injection, Abbott, North Chicago, IL, USA) and Hinkelmann's reagent (Kanto Chemical, Tokyo, Japan), acetylcholine chloride (Ovisot®, Daiichi Pharmaceutical, Tokyo, Japan), D-galactosamine hydrochloride (Wako), lipopolysaccharide (Difco Laboratories, Detroit, MI, USA), [$^3$H]cAMP (20–50 Ci/mmol) and [$^3$H]cGMP (25–50 Ci/mmol) were purchased from NEN Research Products (Boston, MA, USA).

The following chemicals were purchased from Sigma (St. Louis, MO, USA): ovalbumin (Grade III), carbachol, gallamine triethiodide, zymosan A, cyclic 3′,5′-adenosine monophosphate, cyclic 3′,5′-guanosine monophosphate, 5′-nucleotidase (snake venom), calmodulin and aminophylline.

WEB 2086 was kindly provided by Boehringer-Ingelheim (Ingelheim, Germany).

For oral administration, KF19514 was dissolved in distilled water. Theophylline and rolipram were suspended in distilled water. For the in vitro experiments and intravenous administration, KF19514 and aminophylline were dissolved in saline. Rolipram was dissolved in poly(ethylene glycol) 400 or dimethyl sulfoxide, then diluted with saline. Aminophylline (ethylenediamine salts of theophylline) was used for the in vitro experiments and intravenous administration instead of theophylline.

[$^3$H]cAMP and [$^3$H]cGMP were purified by passing them through a DEAE-Sephadex A25 (Pharmacia) column by the method of Kincaid and Manganiello (1988) before use.

### 2.11. Statistical evaluation

The data are expressed as means ± S.E.M. Statistical significance of differences between two groups was determined by means of Student's *t*-test. When more than two means were to be compared, Dunnett's or Steel's multiple range test was used to identify differences among groups and William's multiple range test was used in the case of dose-dependent changes. Differences were accepted as significant at $P < 0.05$.

## 3. Results

### 3.1. Effect on phosphodiesterase

Five isozymes of phosphodiesterase were separated from canine tracheal smooth muscle as Torphy and Cieslinski (1990) have previously reported. The classification of the isozymes of phosphodiesterase followed was that of Beavo et al. (1994), that is phosphodiesterase 1 ($Ca^{2+}$/calmodulin-dependent), phosphodiesterase 2 (cGMP-stimulated), phosphodiesterase 3 (cGMP-inhibited), phosphodiesterase 4 (cAMP-specific) and phosphodiesterase 5 (cGMP-specific). The isozyme fractions were identified as follows. Phosphodiesterase 1 was activated by the addition of $Ca^{2+}$/calmodulin (200 U/ml). Phosphodiesterase 2 was

Table 1
Inhibition of phosphodiesterase isozymes from canine tracheal smooth muscle

| | n | Phosphodiesterase ($IC_{50}$, μM) | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| KF19514 | 3 | 0.27 ± 0.00 | > 10 | > 10 | 0.40 ± 0.01 | > 10 |
| Rolipram | 3 | > 20 | > 20 | > 20 | 1.74 ± 0.13 | > 20 |
| Theophylline | 3 | > 100 | > 100 | > 100 | > 100 | > 50 |

Each value represents the mean ± S.E.M.

activated in the presence of cGMP (10 μM). Phosphodiesterase 3, 4, and 5 were identified by their substrate specificity and with selective inhibitors. Phosphodiesterase 3 was inhibited by milrinone ($IC_{50}$ = 3.0 μM), phosphodiesterase 4 was inhibited by rolipram ($IC_{50}$ = 1.7 μM) (Table 1), and phosphodiesterase 5 was inhibited by zaprinast ($IC_{50}$ = 0.22 μM). Rolipram did not inhibit phosphodiesterase 1, 2, 3 and 5 more than 50% at 20 μM (Table 1). KF19514 produced potent inhibitory effects on phosphodiesterase 4 and phosphodiesterase 1. The $IC_{50}$ values for phosphodiesterase 4 and phosphodiesterase 1 were 0.41 and 0.27 μM, respectively (Table 1). KF19514 did not inhibit phosphodiesterase 2, 3 and 5 more than 50% at 10 μM. Theophylline did not exhibit more than 50% inhibition with any phosphodiesterase isozyme within the dose range tested.

### 3.2. Effects on tracheal smooth muscle in vitro

The effects of KF19514 upon precontracted tracheal smooth muscle strips are shown in Fig. 2. KF19514, rolipram and aminophylline produced a concentration-dependent relaxation of guinea-pig tracheal preparations regardless of stimulant and $EC_{50}$ values (μM) for the antigen-induced contraction were 0.058 ± 0.028, 0.34 ± 0.32 and 16.7 ± 2.22 (mean ± S.E.M.), respectively (Table 2). KF19514 and rolipram produced more potent inhibitory effects on the antigen-induced bronchoconstriction than on

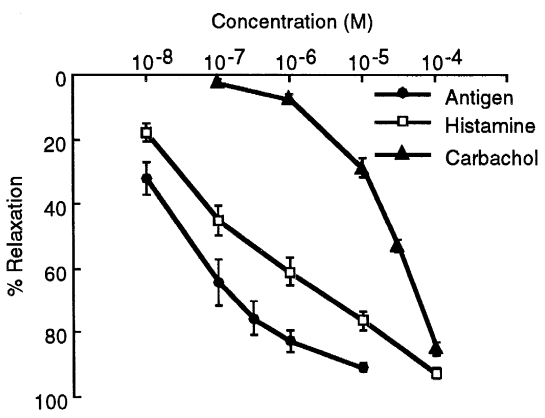


Fig. 2. Relaxant effects of KF19514 on spasmogen-induced contraction in guinea-pig tracheal smooth muscle ($n$ = 5). After induction of contraction with antigen, histamine or carbachol, KF19514 was added cumulatively. Each point represents the mean ± S.E.M.

Table 2
Relaxant effects of KF19514 on isolated guinea-pig trachea

| | n | $EC_{50}$ (μM) | | |
|---|---|---|---|---|
| | | antigen | histamine | carbachol |
| KF19514 | 5 | 0.058 ± 0.028 | 0.44 ± 0.14 | 19.8 ± 1.12 |
| Rolipram | 5–6 | 0.34 ± 0.32 | 0.44 ± 0.14 | 14.3 ± 3.26 |
| Aminophylline | 6–11 | 16.7 ± 2.22 | 33.2 ± 0.14 | 37.7 ± 8.22 |

Each value represents the mean ± S.E.M.

Table 3
Effects on histamine-induced bronchoconstriction

| | n | $ID_{50}$ (mg/kg i.v.) | 95% confidence limits |
|---|---|---|---|
| KF19514 | 5–6 | 0.0028 | (0.0019–0.0041) |
| Rolipram | 6–12 | 0.0079 | (0.0060–0.0107) |
| Aminophylline | 6 | 2.02 | (1.14–3.52) |

$ID_{50}$ values were determined from the percent inhibition.

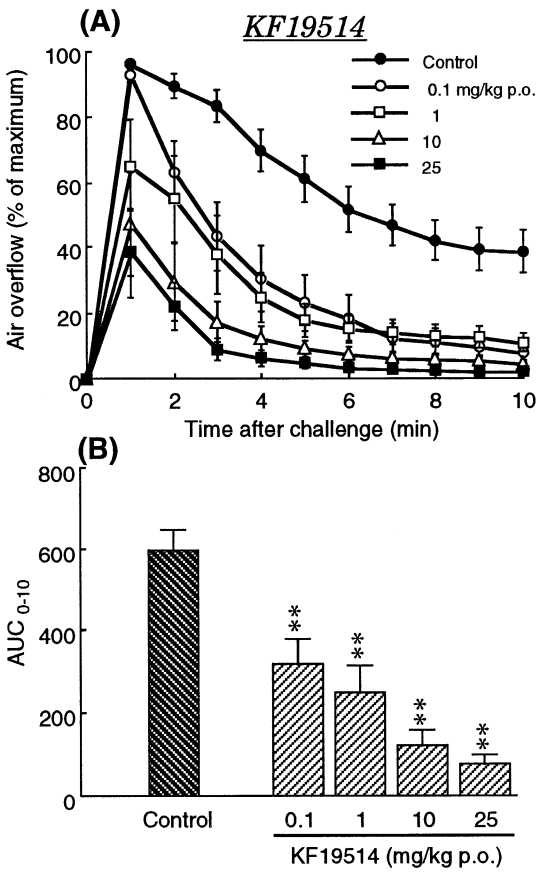


Fig. 3. Effect of KF19514 on anaphylactic bronchoconstriction in anesthetized guinea pigs ($n$ = 6–12). Guinea pigs were passively sensitized with anti-ovalbumin serum 16–24 h before use. KF19514 was administered orally 1 h before ovalbumin challenge. Bronchoconstriction was measured every minute as a percentage of maximum overflow volume (A) and the area under the curve for 10 min ($AUC_{0-10}$) of bronchoconstriction was calculated (B). Results are expressed as means ± S.E.M. ** $P < 0.01$, compared with a control group (Control).

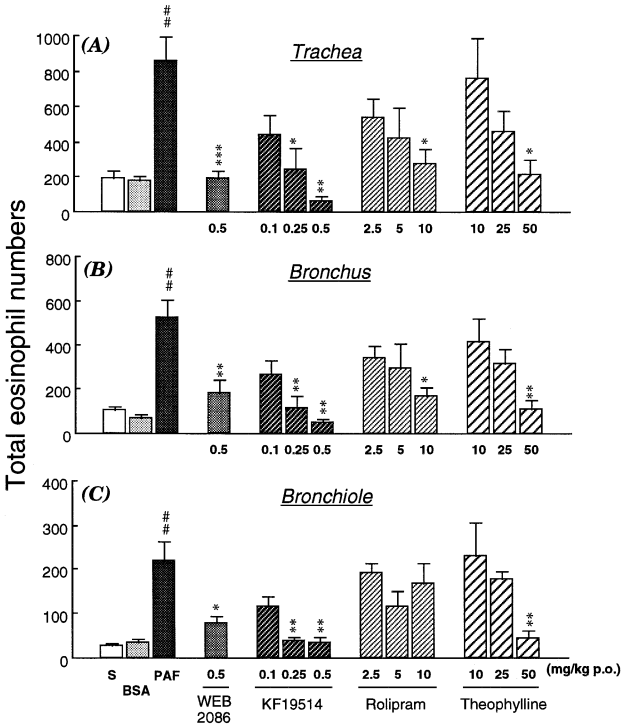


Fig. 4. Effect of KF19514 on PAF-induced eosinophil infiltration in trachea (A), bronchus (B) and bronchiole (C) ($n = 5–6$). Guinea pigs were exposed for 10 min to PAF (1 mg/ml), bovine serum albumin (BSA) or saline (S). The number of eosinophils found in the airway was counted 6 h after PAF exposure. Drugs were administered orally 1 h before PAF exposure. Each bar represents the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with a PAF group (PAF). $^{\#\#}P < 0.01$, compared with a BSA group (BSA).

histamine- or carbachol-induced ones. In contrast to the two other drugs, aminophylline inhibited all the contractions to the same extent.

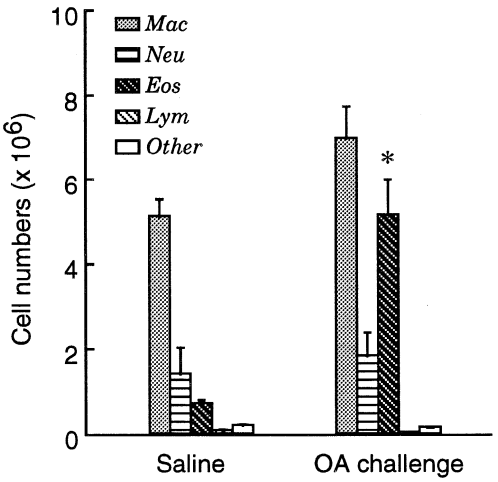


Fig. 5. Differential cell counts in bronchoalveolar lavage fluids from sensitized and saline challenged (Saline), and sensitized and ovalbumin-challenged (OA challenge) guinea pigs ($n = 4–5$). Results are expressed as means ± S.E.M. $^{*}P < 0.05$, compared with a saline group (Saline). Mac = macrophage; Neu = neutrophil; Eos = eosinophil; Lym = lymphocyte; Other = other cell.

Table 4
Effects on anaphylactic bronchoconstriction in anesthetized guinea pigs

| | $n$ | $ID_{50}$ (mg/kg p.o.) | 95% confidence limits |
|---|---|---|---|
| KF19514 | 6–12 | 0.22 | (0.14–0.30) |
| Rolipram | 6–14 | 12.4 | (5.41–28.2) |
| Theophylline | 8–16 | 51.7 | (23.6–113.3) |

The $ID_{50}$ values were determined from $AUC_{0-10}$.

### 3.3. Effects on histamine-induced bronchoconstriction in vivo

Administration (0.0003–0.03 mg/kg i.v.) of KF19514 dose dependently inhibited histamine-induced bronchoconstriction of guinea pigs and the $ID_{50}$ value was 0.0028 mg/kg i.v. (Table 3). Rolipram (0.0003–0.1 mg/kg i.v.) and aminophylline (0.3–10 mg/kg i.v.) also inhibited histamine-induced bronchoconstriction and the $IC_{50}$ values

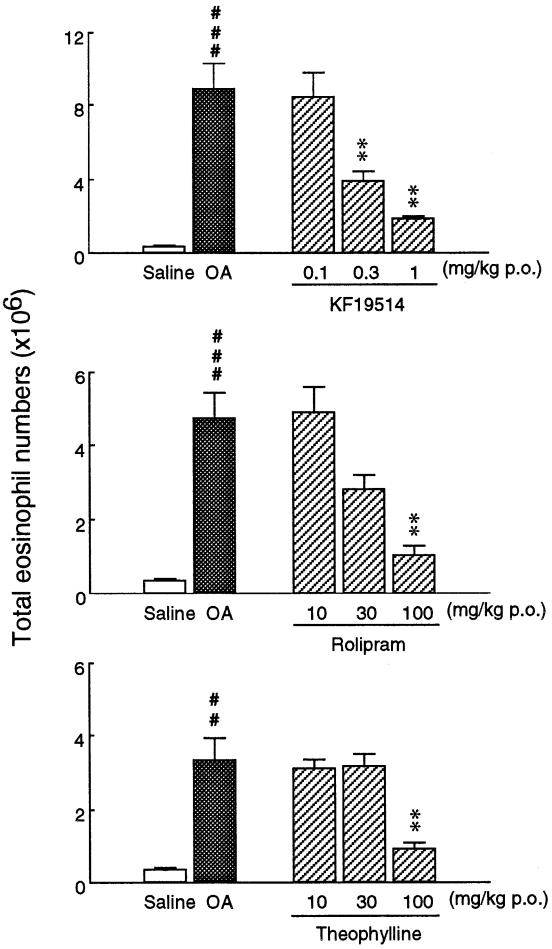


Fig. 6. Effects of KF19514, rolipram and theophylline on eosinophil accumulation in bronchoalveolar lavage fluids from sensitized guinea pigs 24 h after inhalation challenge with aerosolized ovalbumin (OA) ($n = 7–11$). Drugs were administered orally 1 h before challenge. Each bar represents the mean ± S.E.M. $^{**}P < 0.01$, compared with a OA challenged group (OA). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with a saline group (Saline).

were 0.0079 and 2.02 mg/kg i.v., respectively. Vinpocetine, a selective phosphodiesterase 1 inhibitor did not inhibit histamine-induced bronchoconstriction at 0.1 mg/kg i.v. (4% inhibition).

### 3.4. Effects on anaphylactic bronchoconstriction

When ovalbumin (5 μg/kg i.v.) was administered to passively sensitized guinea pigs, anaphylactic bronchoconstriction in control animals occurred approximately 10 s after the ovalbumin injection. The maximum contraction was produced within 2 min, then gradually decreased and remained at approximately 50–70% of the maximum contraction for over 10 min. Oral administration of KF19514 produced a dose-dependent and significant inhibition of the bronchoconstriction (Fig. 3), and the $ID_{50}$ value calculated from $AUC_{0-10}$ was 0.2 mg/kg p.o. (Table 4). Oral administration of rolipram and theophylline also inhibited the anaphylactic bronchoconstriction and the $ID_{50}$ values were 12.4 and 51.7 mg/kg p.o., respectively. Vinpocetine did not exhibit an inhibitory effect at 100 mg/kg p.o. (only 8.9% inhibition).

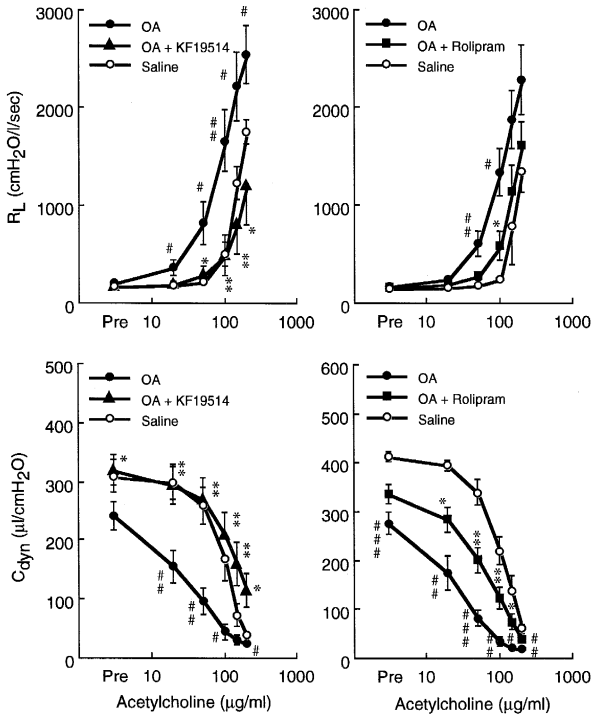


Fig. 7. Effects of KF19514 (0.1 mg/kg p.o.) and rolipram (10 mg/kg p.o.) on the development of hyperreactivity in guinea pigs. The changes in lung resistance ($R_L$) and dynamic compliance ($C_{dyn}$) by exposure to acetylcholine were measured 24 h after inhalation challenge with ovalbumin (OA) in sensitized guinea pigs ($n = 9–10$). Drugs were administered orally 1 h before challenge. Each point represents the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with a OA challenged group (OA). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with a saline challenged group (Saline).

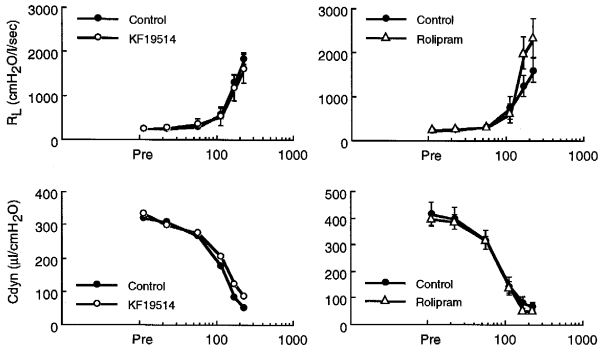


Fig. 8. Effects of KF19514 (0.1 mg/kg p.o.) and rolipram (10 mg/kg p.o.) on lung resistance ($R_L$) and dynamic compliance ($C_{dyn}$) by exposure to acetylcholine 24 h after saline inhalation in sensitized guinea pigs ($n = 5–10$). Drugs were administered orally 1 h before saline challenge. Each point represents the mean ± S.E.M.

### 3.5. Effects on PAF induced eosinophil infiltration

Inhalation of PAF (0.1%, 10 min), as compared to that of vehicle (0.1% bovine serum albumin), induced a significant eosinophil infiltration from the central to peripheral pulmonary airways 6 h later (Fig. 4). There were numerous eosinophil infiltrations under the epithelium of the trachea and bronchus, and around bronchioles in the parenchyma. WEB 2086 (0.5 mg/kg p.o.), a selective PAF receptor antagonist completely suppressed the infiltration of eosinophils. Oral administration of KF19514 produced a dose-dependent and significant inhibition of eosinophil infiltration in the trachea, bronchus and bronchiole, and complete inhibition was produced at 0.5 mg/kg p.o. (Fig. 4). Rolipram and theophylline also inhibited eosinophil infiltration. The inhibitory effect of KF19514 was more potent than that of rolipram and theophylline.

### 3.6. Effects on antigen-induced cellular accumulation in the bronchoalveolar lavage fluid

Ovalbumin inhalation by actively sensitized guinea pigs induced a significant increase in the number of eosinophils in the bronchoalveolar lavage fluid 24 h later (Fig. 5). The number of neutrophils, lymphocytes and alveolar macrophages did not change significantly. Other cells, among which epithelium cells predominated, did not always increase on exposure to the antigen. The oral administration of KF19514 produced a dose-dependent and significant inhibition of eosinophil accumulation in the bronchoalveolar lavage fluid (Fig. 6). Rolipram and theophylline also inhibited eosinophil accumulation in the bronchoalveolar lavage fluid but caused a less potent inhibition than did KF19514. Vinpocetine did not produce an inhibitory effect on eosinophil accumulation at 100 mg/kg p.o. (data not shown).

### 3.7. Effects on antigen-induced hyperreactivity

Ovalbumin inhalation by actively sensitized guinea pigs increased bronchoconstrictor responses to acetylcholine 24

h later. The inhalation of acetylcholine produced a concentration-dependent increase in lung resistance ($R_L$) and decrease in dynamic compliance ($C_{dyn}$). The oral administration of KF19514 (0.1 mg/kg) completely inhibited the development of hyperreactivity (Fig. 7). Significant inhibition was observed in both $R_L$ and $C_{dyn}$. However, KF19514 (0.1 mg/kg p.o.) did not affect the acetylcholine-induced bronchoconstriction 24 h after oral administration (Fig. 8). Rolipram (10 mg/kg p.o.) also inhibited hyperreactivity without the bronchodilator effect.

### *3.8. Effects on tumor necrosis factor-α (TNF-α) production*

Intravenous administration of lipopolysaccharide and D-galactosamine significantly increased the TNF-α content in the mouse serum 1 h after the challenge, while the TNF-α content in normal mouse serum was below the sensitivity limit of ELISA. The TNF-α content in serum of control (vehicle) group was $1.30 \pm 0.22$ ng/ml (mean ± S.E.M., $n = 11$). The oral administration of KF19514 caused a dose-dependent reduction in TNF-α content in serum (Fig. 9) and the $ID_{50}$ value was 0.023 mg/kg p.o. (Table 5). Rolipram and theophylline also inhibited TNF-α production and the $ID_{50}$ values were 1.18 and 12.1 mg/kg p.o., respectively.

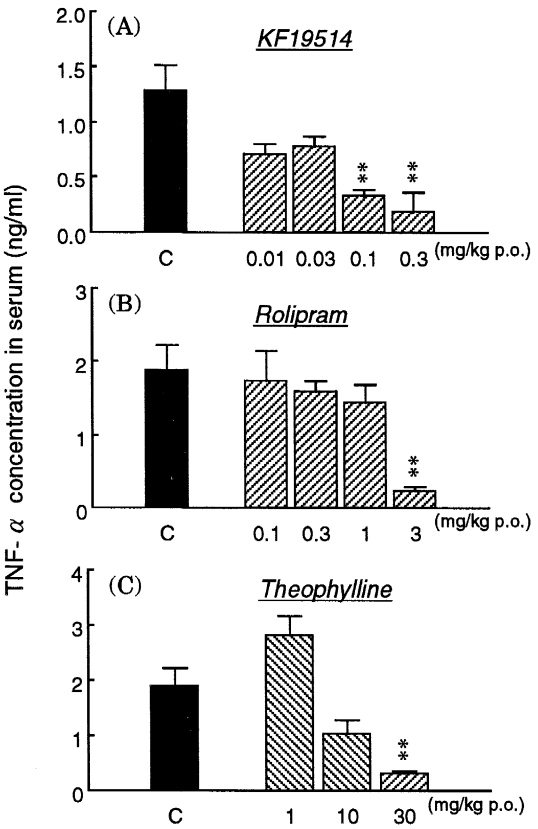


Fig. 9. Effects of KF19514, rolipram and theophylline on TNF-α production induced by lipopolysaccharide in D-galactosamine-sensitized mice ($n = 8$–11). Drugs were administered 30 min before lipopolysaccharide and D-galactosamine injection. Each bar represents the mean ± S.E.M. $^{**}P < 0.01$, compared with a control group (C).

Table 5
Effects on tumor necrosis factor-α (TNF-α) production

| | *n* | $ID_{50}$ (mg/kg p.o.) | 95% confidence limits |
|---|---|---|---|
| KF19514 | 11 | 0.023 | (0.0080–0.067) |
| Rolipram | 8 | 1.18 | (0.148–9.46) |
| Theophylline | 8 | 12.1 | (7.67–19.1) |

## 4. Discussion

Phosphodiesterase 4 inhibitors are well known for their inhibitory effect on bronchoconstriction and inflammation and may be promising potent anti-asthma drugs (Torphy and Undem, 1991; Nicholson and Shahid, 1994). All, including WAY-PDA-641, RP 73401 and CDP 840, are rolipram derivatives. We have investigated the effect of a novel imidazonaphthyridin but non-rolipram derivative, KF19514, on bronchoconstriction and inflammation in guinea pigs and mice. KF19514 inhibited both phosphodiesterase 4 and phosphodiesterase 1, but not phosphodiesterases 2, 3, 5 over $10^{-5}$ M. In contrast to phosphodiesterase 4 inhibitors, phosphodiesterase 1 inhibitors including vinpocetine have not demonstrated an anti-asthmatic effect. In fact, our results indicated that vinpocetine at 100 mg/kg p.o., which appeared to be sufficient to exhibit a pharmacological effect (Debreczeni and Takács, 1976), did not inhibit anaphylactic bronchoconstriction and eosinophil accumulation in bronchoalveolar lavage fluid. However, it remains unclear whether phosphodiesterase 1 inhibition contributes to the bronchodilator effects and the suppression of eosinophil accumulation.

KF19514 induced functional relaxation of precontracted tracheal smooth muscle, in particular, potent relaxation against antigen-induced contraction. It is possible that suppression of mediator release from mast cells, in addition to a direct smooth muscle relaxant effect, contributes to the inhibition of this antigen-induced bronchoconstriction. The effect of KF19514 on mast cell degranulation was not investigated, but rolipram and RO 20-1724 inhibit antigen-induced mediator release from human isolated lung mast cells (Torphy et al., 1992). KF19514 did not exhibit an antagonistic effect on adenosine $A_1$, $A_{2A}$, histamine $H_1$, muscarinic M2, muscarinic nicotine, 5-$HT_{1A}$, 5-$HT_{2A}$, dopamine $D_1$, $D_2$ receptors, $\alpha_1$, $\alpha_2$, $\beta_1$, adrenoceptors over $10^{-5}$ M and the relaxant effect of KF19514 did not change in the presence of propranolol (data not shown). Therefore, the relaxant effect of KF19514 was not a result of specific receptors. Similarly, rolipram and the other phosphodiesterase 4 inhibitors have been demonstrated to produce functional relaxation of airway smooth muscle (Heaslip et al., 1994; Raeburn et al., 1994). It was there-

fore assumed that the relaxant effect of the phosphodiesterase 4 inhibitor was caused by an increasing cAMP content, which is widely accepted to mediate relaxation of airway smooth muscle (Torphy, 1987; Giembycz and Raeburn, 1991).

Both KF19514 and rolipram produced a less potent effect on carbachol-induced contraction than on antigen- or histamine-induced contraction. Similarly, RP-73401 and WAY-PDA-641 also had a less potent effect on carbachol-induced contraction (Heaslip et al., 1994; Raeburn et al., 1994). This might be linked to adenyl cyclase inhibition induced by carbachol through the muscarinic receptor (Torphy et al., 1985). Although preferential inhibition of histamine-induced contraction by rolipram has also been observed in bovine trachea (Shahid et al., 1991), the finding that, in human bronchial smooth muscle, rolipram was equally effective against muscarinic and histamine receptor agonists indicates that different or additional factors are involved (De Boer et al., 1992).

Intravenous administration of KF19514 inhibited histamine-induced bronchoconstriction, and KF19514 caused the most potent inhibitory effect compared to rolipram and aminophylline. In particular, KF19514 ($ID_{50}$ = 0.0028 mg/kg i.v.) was considerably more potent than the non-selective phosphodiesterase inhibitor, aminophylline ($ID_{50}$ = 2 mg/kg i.v.). Oral administration of KF19514 inhibited antigen-induced bronchoconstriction and KF19514 was 60 times and 260 times more potent than rolipram and theophylline, respectively. Although in vivo activity is affected by many factors, such as absorption, metabolism and excretion, KF19514 was the most potent agent, which was consistent with the results in vitro.

The effect of KF19514 on eosinophil infiltration into the airways was investigated for the purpose of studying the anti-inflammatory effect. It is now widely accepted that eosinophilic inflammation is a hallmark of bronchial asthma (Bousquet et al., 1994).

We investigated the effect of KF19514 on the PAF-induced eosinophil infiltration in the airway. It is well known that PAF is a potent chemotactic factor for a wide range of inflammatory cells, particularly eosinophils (Sanjar et al., 1990). The infiltration of eosinophils into lung tissues was exogenously induced by inhalation of PAF. Numerous eosinophils infiltrated from central to peripheral pulmonary airways, all of which was inhibited by treatment with KF19514, with complete inhibition at 0.5 mg/kg p.o.

Eosinophil accumulation in bronchoalveolar lavage fluids was also induced by antigen inhalation in actively sensitized guinea pigs. Antigen inhalation selectively induced eosinophil accumulation in bronchoalveolar lavage fluids. The oral administration of KF19514 suppressed this antigen-induced eosinophil accumulation.

In order to study the mechanism by which KF19514 inhibits eosinophil infiltration into airways, we studied the effect of KF19514 on TNF-$\alpha$ production. TNF-$\alpha$ is one of the cytokines which play a potential role in the pathogenesis of asthma (Kips et al., 1993; Shah et al., 1995). There is evidence that an increased amount of TNF-$\alpha$ is present in the asthmatic airways (Ying et al., 1991; Broide et al., 1992). TNF-$\alpha$ is also recognized to upregulate adhesion molecules including E selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells (Pober et al., 1986; Bevilacqua et al., 1987; Osborn et al., 1989) and is directly responsible for transendothelial migration of inflammatory cells (Ming et al., 1987). Phosphodiesterase 4 inhibitors are now known to suppress the release of TNF-$\alpha$ (Prabhakar et al., 1994; Verghese et al., 1995) and the increase in cAMP inhibits the expression of adhesion molecules elicited by TNF-$\alpha$ (Pober et al., 1993; Panettieri et al., 1995). KF19514 was also demonstrated to suppress the release of TNF-$\alpha$ from human monocytes (Suda et al., 1995). We now investigated the effect of KF19514 on TNF-$\alpha$ production in vivo. KF19514 inhibited lipopolysaccharide-induced TNF-$\alpha$ production in a dose-dependent manner ($ID_{50}$ = 0.023 mg/kg p.o.). This suggests that the mechanism by which KF19514 inhibited eosinophil accumulation in the airway involves in part suppression of TNF-$\alpha$ production.

Airway hyperreactivity is one of the most important pathophysiological features of bronchial asthma. In the present study, lung resistance ($R_L$) and dynamic compliance ($C_{dyn}$) were measured simultaneously to assess airway responsiveness to acetylcholine. It has been shown that $R_L$ is primarily linked to changes in the central airways and $C_{dyn}$ is primarily linked to changes in the peripheral airways (Macklem et al., 1969; Hahn et al., 1976). KF19514 almost completely inhibited the increase in $R_L$ and the decrease in $C_{dyn}$ induced by acetylcholine inhalation at 0.1 mg/kg p.o., a dose at which KF19514 had no direct bronchodilator and/or anti-cholinergic effect. Therefore, it was demonstrated that KF19514 inhibited the hyperreactivity distinct from its bronchodilator effect.

KF19514 inhibited both hyperreactivity and eosinophil accumulation, but at different doses. For example, KF19514 did not suppress eosinophil accumulation at 0.1 mg/kg p.o., at which dose the hyperreactivity was completely inhibited. Similarly, rolipram inhibited the hyperreactivity and eosinophil accumulation at different doses. This was first observed for rolipram by Santing et al. (1995). Lagente et al. (1993) and Boichot et al. (1993) also reported a dissociation between the number of eosinophils in the bronchoalveolar lavage fluids and the development of hyperreactivity in this model. It was reported that bronchial hyperreactivity was definitely not related to the number of eosinophils in the bronchoalveolar lavage fluids but was closely related to eosinophil activity (Santing et al., 1994). The inhibitory effect of KF19514 on hyperreactivity could result partly from suppression of eosinophil activation, because KF19514 significantly inhibits the release of pep-

tide-leukotrienes from guinea-pig eosinophils stimulated with Ca ionophore A23187 (Manabe et al., 1995). In addition, the effect might be related to the inhibition of TNF-$\alpha$ production as previously mentioned, since TNF-$\alpha$ directly induces hyperreactivity independent of cellular accumulation (Wheeler et al., 1990; Kips et al., 1992).

Recently, dry powder formulation of agents for lung diseases such as asthma has been considered to be a useful technique, advantages of which are rapid onset of action and less systemic effect, especially side-effects. Therefore, we are now evaluating the effect of KF19514 on bronchoconstriction and pulmonary inflammation, and found that KF19514 produced a significant anti-asthmatic effect even on inhalation (Manabe et al., manuscript in preparation).

In conclusion, KF19514 given orally produced a potent bronchodilator effect and inhibited eosinophil infiltration into the lung, which might be primarily a result of phosphodiesterase 4 inhibition. These results suggest that KF19514 could be of significant value in the treatment of asthma.

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