

Effect of pranlukast on bronchial inflammation in patients with asthma

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Summary

Background Pranlukast (8-[p-(4-phenylbutoxy) benzol] amino-2-[tetrazol-5-yl]-4-oxo-4H-1-benzopyran hemihydrate), a selective cysteinyl leukotriene receptor antagonist, has been reported to exhibit not only antileukotriene activity but also pharmacological activity including antieosinophilic effects.

Objective This study was designed to investigate whether the antiasthmatic activity of pranlukast is associated with a reduction in eosinophilic inflammation.

Methods A double-blind, randomized, crossover design was used. Subjects received 225 mg of pranlukast or placebo orally twice daily for 4 weeks and then, after a washout period of at least 4 weeks, crossed over to receive the alternative treatment. We assessed the effects of pretreatment with pranlukast on bronchoconstriction precipitated by inhalation of methacholine in 32 adult patients with mild or moderate bronchial asthma; those who were in stable clinical condition were allocated to this study. Blood and sputum samples were taken the morning of the methacholine provocation test. Eosinophil counts and measurement of eosinophilic cationic protein (ECP) were performed.

Results After the 4 weeks of treatment with pranlukast, patients' symptoms, blood eosinophils, serum ECP, sputum eosinophils, and sputum ECP were significantly decreased. Furthermore, values of PC₂₀-methacholine significantly improved in the treatment with pranlukast.

Conclusion Our results suggest that pranlukast has an anti-inflammatory effect on bronchial eosinophilic infiltration. This study raises further interesting therapeutic possibilities and argues for further trials of new approaches to the treatment of bronchial asthma.

Keywords: Bronchial asthma, bronchial eosinophilic inflammation, eosinophilic cationic protein, Pranlukast

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Introduction

Pranlukast (8-[p-(4-phenylbutoxy) benzol] amino-2-[tetrazol-5-yl]-4-oxo-4H-1-benzopyran hemihydrate), a selective receptor antagonist of cysteinyl leukotrienes (LTC₄, LTD₄, and LTE₄), is now widely used in the treatment of asthma. Many clinical studies have shown that pranlukast is well tolerated and is effective in reducing bronchial hyperresponsiveness and

allergen-induced bronchoconstriction [1]. The anti-asthmatic effect of pranlukast may be rendered not only by antileukotriene activity, but also by pharmacological activity including antieosinophilic effects. An article described pranlukast's effect in reducing eosinophilic inflammation in diseases whose pathogenesis does not involve cysteinyl leukotrienes [2]. Also, other anticysteinyl leukotriene receptor antagonists have shown reductions in eosinophils in sputum and blood [3–5], and similar results are seen with the leukotriene synthesis inhibitor zileuton [6].

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We know that bronchial hyperresponsiveness is a characteristic of bronchial asthma, and it has been emphasized that airway inflammation associated with eosinophilic infiltration plays an important role in bronchial hyperresponsiveness in patients with asthma [7]. Oddera *et al.* reported that bronchial eosinophil recruitment and activation may occur even in mild to moderate stable asthma and that bronchial epithelium damage and airway responsiveness may be partially associated with the eosinophilic inflammatory reaction [8]. There is also much evidence that bronchial asthma is characterized by chronic recruitment of eosinophils to the airways, and eosinophils play an important role in bronchial epithelial damage in asthma by releasing cationic proteins [9–11]. Allergen exposure in atopic asthmatic patients is associated with recruitment and activation of eosinophils in the airways. Eosinophils release toxic products, including the eosinophil cationic protein (ECP), which damage bronchial structures, and increase bronchial hyperresponsiveness once they are activated [12]. Serum ECP is thought to be a useful marker of eosinophilic inflammation in bronchial asthma [9–11,13].

The aims of the present study were to confirm previous observations of pranlukast's anti-eosinophilic activity and other activities related to bronchial inflammation and to examine whether the protective activity of this drug against asthma is associated with a reduction in the eosinophilic inflammation that contributes to the pathogenesis of asthma.

Materials and Methods

Subjects

We studied 32 adult patients with allergy-induced asthma (Table 1). All patients were nonsmokers (never smokers only), neither were aspirin intolerant nor COPD, and all had mild or moderate asthma diagnosed according to the criteria of the American Thoracic Society [14]. The patients were in a stable condition and had been free of symptoms of respiratory infection for at least 6 weeks.

Patients using oral or inhaled corticosteroids, any anti-leukotriene drug including pranlukast, and chemical mediator inhibitors such as cromolyn sodium were excluded. All patients used a metered-dose inhaler of β -stimulants on demand and/or theophylline to control their asthma. The study was conducted according to the ethical standards of our medical centre, which required informed consent from each patient.

Study design

A double-blind, randomized, crossover design was used. Subjects were randomly assigned to receive orally a 4-week course of two daily doses of 225 mg of pranlukast (ONO

Pharmaceutical Co., Ltd, Osaka, Japan) or a dose of placebo that was identical in appearance. After a washout period of at least 4 weeks, the subjects crossed over to receive the alternative treatment for 4 weeks.

FEV₁, FVC, and methacholine provocation testing were performed. Blood and induced-sputum, differential and total cell counts were determined before and after each 4-week treatment course.

Blood count and serum eosinophilic cationic protein (ECP) measurement

Blood samples for eosinophil counting and measurement of ECP were taken between 8 and 9 a.m. on the day of methacholine provocation testing [15]. Blood was collected in heparinized tubes by direct venipuncture, and blood smears were made for differential staining (Diff quick; American Scientific Products, McGawPark, IL, USA). Differential cell counts were calculated as the mean value of two slides, with 300 cells counted per slide. The total leucocyte count was determined with a haemocytometer (Neubauer Chamber: Hausser Scientific, Blue Bell, PA, USA), and cell populations were expressed as number of cells per millilitre of blood.

To avoid interassay variations, all ECP measurements were performed in the same run. Blood samples of 4 mL were collected in a SST tube (Becton Dickinson, Mountain View, CA, USA), stored for 60 min at 25 °C, and subsequently centrifuged at 1300 g for 10 min at 4 °C. Serum was stored at –80 °C until ECP levels were measured with an ECP RIA (radioimmunoassay) kit (Pharmacia Diagnostics, Uppsala, Sweden). The detection limit of this assay is 2.0 µg/L.

Serum IgE measurement

Serum samples collected from patients at the same time as eosinophil counts and measurement of ECP were screened for total IgE with the FEIA-CAP system (Pharmacia Inc., Columbus, OH, USA) according to the manufacturer's instructions.

Sputum analysis

Sputum was induced and processed as described by Popov *et al.* [15] Subjects inhaled 3%, 4%, and 5% saline for 7 min each. The induction was stopped when adequate samples were obtained or if the FEV₁ dropped by 20% from baseline. Cell plugs with few or no squamous epithelial cells were selected from the sample with an inverted microscope, separated from saliva, and weighed. Samples were aspirated in twice their volume of 0.1% dithiothreitol (Sputolysin, Calbiochem Corp., San Diego, CA, USA) and twice

Table 1. Patient characteristics at entry into the study of pranlukast for treatment of bronchial inflammation in patients with asthma

Patient No.	Sex	Age (year)	Atopy* (PST positive)	FEV ₁ (% pred)	FVC (% pred)	Eosinophils† (%)	Serum IgE (RIST, U/mL)	Medication‡†
1	M	38	G, HD	77	91	8	37	B
2	M	56	G, DM	73	88	14	340	T, B
3	M	29	F, G, HD	70	101	9	245	T, B
4	M	45	G, HD	74	95	7	102	T, B
5	M	41	HD, DM, RW	89	92	10	89	B
6	M	46	CD, F, HD	78	96	4	63	T, B
7	M	35	F, G, HD	71	100	12	116	B
8	M	51	B, F	94	79	9	77	B
9	M	37	DM, G, HD	71	97	11	133	T, B
10	M	21	CD	75	104	4	37	B
11	M	52	G, HD	77	87	15	340	T, B
12	M	24	CP, HD	69	106	14	245	T, B
13	M	44	DM, G, HD	73	96	8	102	B
14	M	43	F, G	88	93	4	89	B
15	F	38	G, DM	76	94	6	63	T, B
16	F	37	CD, D, HD	78	91	8	116	T, B
17	F	58	HD	82	82	10	77	B
18	F	47	G, HD	76	96	19	466	B
19	F	29	DM, G, HD	87	103	26	1600	B
20	F	47	CP, RW	76	92	17	260	B
21	F	38	F, G, HD	70	97	8	132	T, B
22	F	42	HD, DM	84	90	9	151	B
23	F	53	DM, G, HD	76	88	5	86	T, B
24	F	45	G	72	96	6	54	T, B
25	F	36	G, H	76	97	17	466	T, B
26	F	22	CP	87	87	13	475	B
27	F	43	DM, G, HD	76	99	8	260	T, B
28	F	39	G, HD	70	96	4	132	T, B
29	F	45	DM, G, HD	84	94	7	151	B
30	F	55	CD, F	76	95	2	386	T, B
31	F	45	G, HD	72	96	18	568	T, B
32	F	39	F, G, HD	70	110	4	132	T, B
mean ± SD		41 ± 9.4		77 ± 6.5	95 ± 6.5	9.9 ± 5.4	237 ± 289	

* B; buckwheat; CD, cat dander; CP, cedar pollen; D, dog; DM, dust mite; F, fungus; G, grass; H, horse; HD, house dust; RW, ragweed.

† Peripheral blood eosinophils. ‡ T, theophylline; B, inhaled B₂-agonist on demand. All patients were diagnosed with mild or moderate asthma according to American Thoracic Society's criteria. The patients were in stable condition and had been free of symptoms of respiratory infections for at least 6 weeks.

their volume of Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY, USA). The cell suspension was filtered through nylon gauze (mesh size is 52 µm; BNSH Thompson, Scarborough, ON, Canada) to remove debris, then centrifuged at 1300g for 10 min at 4 °C. Supernatants were collected and stored at -80 °C for fluid-phase measurements of ECP.

The total cell count was determined with a haemocytometer (Neubauer Chamber) and expressed as the number of cells per millilitre of sputum. Cells were resuspended in DPBS at a concentration of 0.75–1.0 × 10⁶/mL.

Cell preparations for cytocentrifugation were made on glass slides from 50 µL of cell suspension and subjected to cytocentrifuge (Shandon III, Shandon Southern Instruments, Sewickley, PA, USA), at 300g for 5 min. Differential cell counts were obtained from the mean of two slides stained with Diff Quick with 400 cells counted per slide. Cell types were quantified as number of cells per milliliter of sputum. Cell preparations for cytocentrifugation were made on apex-coated slides and fixed for 10 min in periodatelysine-paraformaldehyde for immunocytochemical staining for ECP.

Methacholine provocation testing

Bronchial responsiveness was evaluated by methacholine provocation according to the standard method of the Japanese Society of Allergology [16,17]. This test employed methacholine chloride (Sigma, St. Louis, MO, USA), dissolved in saline to make solutions of 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 mg/mL administered by a jet nebulizer driven by compressed air (Nihon Shoji Co., Ltd. Osaka, Japan).

All patients were given a provocation test after a 4-week treatment course with either pranlukast or placebo in a double-blind, randomized manner. Thereafter subjects were crossed over to receive alternative treatment, and following an identical procedure at the same time of day. To avoid the acute-phase effect of pranlukast, each provocation test was performed before taking pranlukast in the morning. Each study day began with the measurement of FEV₁ and FVC for baseline, followed by inhalation of nebulized saline to rule out a nonspecific obstructive reaction; FEV₁ was measured 5 min before and 5, 10, and 15 min after completion of inhalation. If FEV₁ did not fall more than 10%, the subject entered the main part of study.

Methacholine was inhaled for 2 min by tidal breathing with a nose clip, immediately followed by spirometry. Methacholine of increasing concentration was successively inhaled until FEV₁ fell by 20% or more from baseline. The measured values were plotted on semilogarithmic graph paper, and the methacholine provocative concentration producing a 20% fall in the FEV₁ was calculated as PC₂₀-methacholine. The provocation test was terminated by the administration of an inhaled bronchodilator if FEV₁ fell by more than 35% from baseline. The higher of the first two satisfactory recordings was taken for analysis.

Symptom score

To evaluate the effect of pranlukast on the improvement of symptoms in asthmatics, symptoms were assessed weekly

by physicians using four grades: 0 = asymptomatic more than 4 days/week; 1 = mild symptom (aware of symptoms but easily tolerated) more than 4 days/week and nocturnal asthma symptoms less than 2 days/week; 2 = moderate symptom (discomfort enough to interfere with daily activity) more than 4 days/week and/or nocturnal asthma symptoms more than 2 days/week; and 3 = severe attack (unable to perform usual activity) more than 4 days/week and/or nocturnal asthma symptoms almost everyday. This method is modelled after that of Oosaki *et al.* [18].

Statistical Analysis

Statistical evaluation employed StatView J-4.02 for Macintosh (Abacus Concepts, Inc., Berkeley, CA, USA). All PC₂₀ values were logarithmically transformed before analysis, and summary statistics were expressed as mean $\pm$ SD. Student's paired *t*-test was used to compare mean differences. Wilcoxon's signed-rank test was used for comparison of the two treatment groups at the same time point regarding FEV₁ and ECP values. A *P*-value less than or equal to 0.05 (two-sided) was considered significant.

Results

After the 4 weeks of treatment with pranlukast, none of the subjects reported any adverse effect, nor could they discriminate between pranlukast and placebo. The FEV₁ was significantly improved by administration of pranlukast after 4-weeks of each treatment ($P \leq 0.05$, Table 2).

Twenty-seven of 32 subjects improved in general symptoms, such as nocturnal cough, wheezing, and severity or frequency of asthmatic attack. Other subjects felt no change in their symptoms, since their conditions were stable. No subject got worse than before the beginning of this study. The symptom score significantly decreased after treatment with pranlukast (Table 2, 0.74 ± 0.25 after pranlukast vs 1.66 ± 0.27 after placebo, $P < 0.001$).

Table 2. Bronchial responses as measured by FEV₁ and FVC and symptom score at baseline after 8 weeks of treatment with pranlukast or placebo

	Mean $\pm$ SD (%) of indicated parameter			
	Placebo		Pranlukast	
	Before	After	Before	After
FVC	95.0 $\pm$ 6.5	94.1 $\pm$ 7.8	96.0 $\pm$ 7.0	97.2 $\pm$ 6.8
FEV ₁	77.0 $\pm$ 6.5	76.8 $\pm$ 6.7	77.1 $\pm$ 4.7	86.9 $\pm$ 4.1*
Symptom score	1.68 $\pm$ 0.38	1.68 $\pm$ 0.43	1.66 $\pm$ 0.27	0.74 $\pm$ 0.25***

The value marked with asterisks were significantly greater than values at after placebo. * $P < 0.05$, *** $P < 0.001$.

Table 3. Comparison of parameters measured before and after 8 weeks' treatment with pranlukast in patients with bronchial asthma

	Mean $\pm$ SD of indicated parameter			
	Placebo		Pranlukast	
	Before	After	Before	After
<i>Blood</i>				
Eosinophils ($\times 10^4/\text{mL}$)	44.5 $\pm$ 6.9	43.8 $\pm$ 9.7	44.7 $\pm$ 8.3	12.3 $\pm$ 2.4**
Neutrophils ($\times 10^4/\text{mL}$)	445 $\pm$ 56	449 $\pm$ 63	458 $\pm$ 44	426 $\pm$ 69
Serum ECP (mg/L)	16.2 $\pm$ 6.9	15.1 $\pm$ 7.1	16.3 $\pm$ 6.6	3.6 $\pm$ 1.4*
Serum IgE (IU/L)	242 $\pm$ 312	266 $\pm$ 302	237 $\pm$ 289	228 $\pm$ 307
<i>Sputum</i>				
Eosinophils ($\times 10^4/\text{mL}$ sputum)	90 $\pm$ 30	88 $\pm$ 36	93 $\pm$ 29	12 $\pm$ 5*
Neutrophils ($\times 10^4/\text{mL}$ sputum)	271 $\pm$ 70	280 $\pm$ 72	266 $\pm$ 78	199 $\pm$ 85
ECP (mg/L sputum)	1.9 $\pm$ 0.5	2.0 $\pm$ 1.0	1.9 $\pm$ 0.5	0.4 $\pm$ 0.1*

All values marked with asterisks were significantly greater than values at after placebo * $P < 0.05$, ** $P < 0.01$.

Blood eosinophils, serum ECP, sputum eosinophils, and sputum ECP were significantly decreased (Table 3) by pranlukast. On the other hand, the number of blood and sputum neutrophils did not change significantly.

On methacholine provocation day, all subjects were followed for longer than 120 min after provocation test. After pretreatment with pranlukast or placebo, challenge with methacholine caused an obstructive reaction in all patients. Data for PC_{20} -methacholine in all patients are displayed in Fig. 1. In the provocation challenge with methacholine, values of PC_{20} -methacholine significantly improved after pranlukast. Mean log PC_{20} -methacholine was 2.91 ± 0.52 after pranlukast, vs 2.54 ± 0.56 after placebo ($P < 0.01$).

In the present study, after 4-weeks of treatment with either pranlukast or placebo, there is no significant association between the improvement of PC_{20} -methacholine and ECP values, or between the decreasing of eosinophils and the improvement of PC_{20} -methacholine. In addition, Wilcoxon's signed-rank test showed no significant correlation between the maximum falls in FEV_1 or the concentration of methacholine inhaled and the corresponding maximum decrease in ECP values after placebo. All the data among before placebo, after placebo, before pranlukast were not different significantly.

Discussion

Bronchial asthma is widely recognized as a chronic inflammatory disease [19]. Leukotrienes are potent pro-inflammatory mediators that appear to contribute to pathophysiological features of asthma. Specifically, cysteinyl leukotrienes contract airway smooth muscle, increase

microvascular permeability, stimulate mucus secretion, decrease mucociliary clearance, and appear capable of recruiting eosinophils into the airways [20]. Our results showed that 4 weeks treatment with pranlukast improved patients' symptom score, baseline of FEV_1 , bronchial

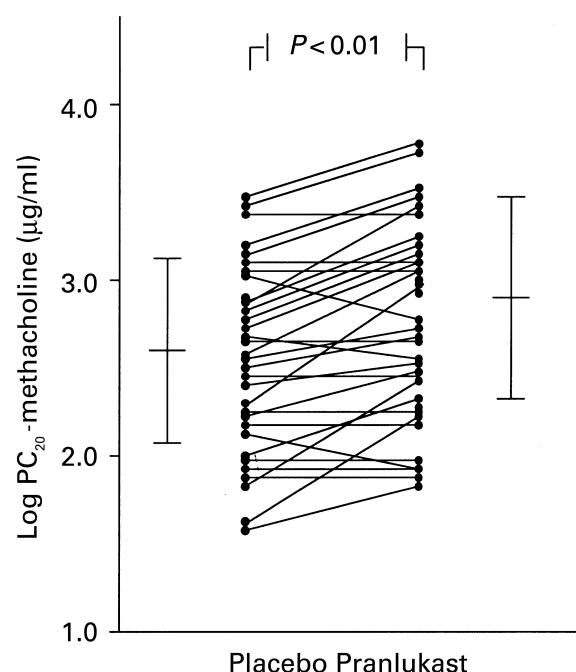


Fig. 1. Individual results of PC_{20} -methacholine at baseline and after treatment with pranlukast or placebo. Both results are shown as the mean, with the bar indicating SD. All PC_{20} values were logarithmically transformed before analysis, and summary statistics were expressed as mean $\pm$ SD

hyperresponsiveness with reduction of the number of eosinophils in blood and sputum, and the value of ECP with other kinds of cysteinyl leukotriene antagonists as previously described [3–6]. It is therefore likely that cysteinyl-leukotriene blockade is the common mechanism of the antieosinophilic effects of all cysteinyl leukotriene antagonists including pranlukast.

There have been reports that some cases of Churg–Strauss syndrome, a granulomatous, necrotizing vasculitis with eosinophilia, is associated with the use of cysteinyl leukotriene antagonists [21,22]. All the patients in whom Churg–Strauss syndrome developed were patients with asthma who were taking cysteinyl leukotriene antagonists and were being weaned from oral corticosteroids. After discontinuation of cysteinyl leukotriene antagonist therapy and administration of systemic corticosteroids the patient's symptoms reversed rapidly and there was prompt resolution of the pulmonary infiltrates. The cysteinyl leukotriene antagonists are thought to mask the symptoms of Churg–Strauss syndrome. This is an evidence showing that cysteinyl leukotriene antagonists modify eosinophils infiltration.

Eosinophils are among the most important components of bronchial inflammation and bronchial hyperresponsiveness [9–11]. Laitinen *et al.* showed that leukotriene LTE_4 elicited an increase in the numbers of eosinophils and neutrophils in the lamina propria of the airway mucosa 4 h after inhalation in four asthmatic subjects [23]. The numbers of eosinophils were, on average, 10-fold greater than those of neutrophils. They also reported there was no significant change in numbers of lymphocytes, plasma cells, mast cells, or macrophages. Pro-inflammatory effects of cysteinyl leukotrienes may contribute significantly to their influential role in the overall pathophysiology of asthma associated with eosinophilic infiltration. Takeda *et al.* showed that neither the presence of mast cells nor IgE mast cell activation is required for the development of bronchial inflammation and bronchial hyperresponsiveness in mice sensitized to allergen via the intraperitoneal route and challenged via the airways, but eosinophils are necessary [24]. Jatakanon *et al.* found significant correlations between exhaled nitric oxide and bronchial hyperresponsiveness, between exhaled nitric oxide and sputum eosinophil counts, and between sputum eosinophil counts and bronchial hyperresponsiveness. Furthermore, they concluded that the relationship among sputum eosinophils, exhaled nitric oxide, and bronchial hyperresponsiveness pointed to the potential use of eosinophils in induced sputum and exhaled nitric oxide to monitor the severity of asthma [25]. Other studies showing that cysteinyl-leukotriene cause specific migration of eosinophils *in vitro* [26], and into the lung after inhalation support the view that leukotriene blockade wholly explains the antieosinophilic effects common to this class of drug [23].

The antieosinophilic effects might be related to antibronchial inflammatory effect of all anticysteinyl leukotriene drugs including pranlukast.

Serum ECP is thought to be a useful marker of eosinophilic inflammation in bronchial asthma, as mentioned above [9–11,13]. ECP has been reported to elevated in bronchoalveolar lavage fluid, to play an active secretory role of the eosinophil in the late-phase asthmatic reaction, and play an active role in the late-phase asthmatic reaction [27]. Release of ECP and other granular proteins may contribute to epithelial damage and inflammation and thereby to an increase in bronchial hyperresponsiveness [28–30]. In this study, we showed that pranlukast, a selective receptor antagonist of type 1 cysteinyl leukotrienes, markedly inhibits blood and sputum ECP level. This effect is not associated with the effect of cysteinyl leukotriene to eosinophils, because pranlukast does not inhibit the production of cysteinyl leukotrienes. Pranlukast may have another pharmacological effects besides the inhibition of cysteinyl leukotriene receptor. In addition, we investigated that induced sputum analysis can be used to evaluate this disease and may prove to be a useful tool for monitoring asthma therapy.

This study raises further interesting therapeutic possibilities and supports the used for further trials of new approaches to the treatment of bronchial asthma.

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