Effect of a 5-lipoxygenase inhibitor on leukotriene generation and airway responses after allergen challenge in asthmatic patients

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Abstract

The effect of a single oral dose (800 mg) of zileuton (A-64077), a specific 5-lipoxygenase inhibitor, on the early and late airway responses to inhaled allergen was studied in a randomised, double blind, placebo controlled, and crossover trial in nine subjects with atopic asthma. Leukotriene generation was also assessed in vivo by measuring urinary leukotriene (LT) E_4 excretion, and ex vivo by measuring calcium ionophore stimulated whole blood LTB_4 production. Zileuton almost completely inhibited ex vivo LTB_4 production but reduced urinary excretion of LTE_4 by only about half. There was a trend for the early asthmatic response to be less on the day of zileuton treatment, but this did not reach statistical significance (p = 0·08). The zileuton induced reduction in maximum fall in FEV_1 in the early asthmatic response was, however, significantly related to the reduction in urinary LTE_4 excretion (r = 0·8), but not to the reduction in LTB_4 generation ex vivo. There was no significant change in the allergen induced late asthmatic response, or in the increase in airway responsiveness to methacholine following antigen. The results provide some support for the hypothesis that the cysteinyl leukotrienes have a role in the allergen induced early asthmatic response. More complete in vivo inhibition of 5-lipoxygenase may be needed to produce a significant reduction in airway response to allergen challenge.

The leukotrienes, products of the 5-lipoxygenase pathway of arachidonic acid metabolism, are thought to be important mediators in the pathogenesis of asthma because their biological activities produce changes that are similar to those seen in asthma.[1-3] The cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, are potent bronchoconstrictor agents in normal and asthmatic subjects,[4-6] and LTE_4 has been reported to increase airway hyper-responsiveness.[7] LTB_4 is a potent chemotactic agent for leucocytes,[8] and may be important in mediating the inflammatory process in asthmatic airways. LTD_4 can increase mucus production in human airway preparations[9] and airway microvascular leakage in animals.[10] Several clinical studies with cysteinyl leukotriene antagonists in asthmatic subjects have

shown a slight reduction in the early asthmatic response to inhaled allergen[11 12] and in cold air induced bronchoconstriction.[13] The compounds studied were relatively weak cysteinyl leukotriene antagonists, however, and they would in addition leave the effects of the dihydroxy acid LTB_4 unopposed. An alternative approach is to inhibit the 5-lipoxygenase enzyme to reduce synthesis of all the leukotrienes. Several different classes of 5-lipoxygenase inhibitors have been found to be effective in animal models of asthma.[14-16] No significant reduction of allergen induced bronchoconstriction was, however, proposed by piriprost[17] or nafazatrom[18] in two clinical studies in man—but nafazatrom was found not to prevent ex vivo leukotriene production in man after oral dosing despite having 5-lipoxygenase inhibitory activity in vitro (the activity of piriprost in man was not studied).

After exposure to inhaled allergen there is an early and late bronchoconstrictor response in asthmatic subjects and an increase in airway responsiveness. Several mediators, including the leukotrienes, are generated during antigen challenge and may be important in mediating the airway responses. Zileuton, a hydroxamic acid 5-lipoxygenase inhibitor,[19] has been shown to inhibit calcium ionophore stimulated human neutrophil production of LTB_4 in man ex vivo after oral dosing,[20] and is effective in inhibiting allergen induced contraction of tracheal smooth muscle in the guinea pig.[21] We have investigated the efficacy of zileuton (800 mg) on the generation of cysteinyl leukotriene in vivo (assessed by urine LTE_4 concentration) induced by inhaled allergen and on the generation of LTB_4 ex vivo (by calcium ionophore stimulated whole blood) in asthmatic subjects. We assessed the inhaled allergen induced airway responses of the early and late asthmatic response and changes in airway responsiveness.

Methods

SUBJECTS

Eleven non-smoking men with atopic asthma (mean age 28, range 19–44 years) with an FEV_1 above 70% predicted were recruited (table). Subjects were having inhaled salbutamol only, apart from one who was also inhaling steroids and taking oral theophyllines. All subjects had been shown to have a dual asthmatic response to allergen on screening. None had had an upper respiratory tract infection or exacerbation of asthma

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within six weeks of starting the study. Non-steroidal anti-inflammatory drugs were not allowed during the study period. The study was approved by the ethics committee of the National Heart and Lung Hospitals, and fully informed written consent was obtained from each subject.

Subjects had to be healthy at the physical examination and to have normal results in biochemical and haematological tests and a normal electrocardiogram before entry into the study. These were repeated during the study and at the end to assess the safety and tolerability of zileuton.

STUDY PROTOCOL

Allergen challenge

On a screening day the atopy of each subject was confirmed by obtaining a positive skin-prick test response to either *Dermatophagoides pteronyssinus* or mixed grass pollen allergen extracts (Pharmacia, Milton Keynes) dissolved in normal saline. Subjects first inhaled the diluent, aerosolised with a Wright nebuliser (volume 2 ml, flow rate 7 l/min, output 0.2 ml/min), through a face mask with open mouthed tidal breathing for two minutes, and FEV_1 was then measured for 15 minutes to exclude a bronchoconstrictor response to diluent. Cumulative doses of allergen were then inhaled in the same way to identify subjects with a dual asthmatic response. The initial allergen concentration used for inhalation was that which caused a 2–3 mm diameter weal in the skinprick test. Doubling concentrations of allergen were inhaled until an early asthmatic response (fall in FEV_1 of at least 20% of the post-diluent value) was seen. No further allergen was then given, FEV_1 was measured up to eight hours to detect any late asthmatic response, which was defined as a fall in FEV_1 of at least 15% of post-diluent value. If a dual response was seen, the final allergen dose reached was used on the two allergen challenge study days. Of the 17 subjects screened, six subjects with an isolated early asthmatic response were not enrolled into the main study.

Two to three weeks later subjects returned for the first allergen challenge study. Zileuton (800 mg) or matching placebo was ingested in the morning, and allergen challenge was performed three hours later. FEV_1 was measured

every 10 minutes for the first hour after challenge, and then hourly for the next seven hours. At least two weeks later subjects returned for the second study to continue with an identical protocol provided that the baseline FEV_1 was within 15% of that on the first study day. If not, they were asked to return for another study day.

FEV_1 was measured with a dry bellow spirometer (Vitalograph, Buckingham) with the subject sitting upright. The mean of three good expiratory efforts was taken. Before each test subjects rested in a warm room for at least 15 minutes.

Non-specific airway responsiveness was assessed according to the method of Cockcroft *et al*[22] on the day before and after each allergen test day, at the same time of the day for each subject. The provocative dose of methacholine needed to reduce FEV_1 by 20% of the post-diluent value (PC_{20} FEV_1) was determined. Methacholine was inhaled in the same way as allergen.

Assessment of 5-lipoxygenase inhibition

5-Lipoxygenase activity after allergen challenge was assessed by measuring urinary LTE_4 excretion.[23] Immediately before challenge subjects emptied their bladder and urine was then collected for four hours. Urine volume and pH were measured and aliquots of 30 ml were stored at $-70°C$. LTE_4 was extracted from the urine by reverse phase high performance liquid chromatography, tritiated LTE_4 being used as an internal standard,[24] and measured by radioimmunoassay (Amersham International, Amersham). The efficacy of zileuton was also assessed by calcium ionophore stimulated whole blood LTB_4 production ex vivo. Three and a half, four, five, seven, nine, and 10 hours after it was given 5 ml of whole blood was incubated with 5 μl of 50 mM calcium ionophore for 15 minutes and centrifuged, and the supernatant was stored at $-70°C$. LTB_4 was extracted by high performance liquid chromatography, and assayed by radioimmunoassay.

Drug level

Plasma level of drug was measured at 3.5, 4 and 5 hours post-dose on both placebo and active days. Zileuton was extracted by high performance liquid chromatography and measured by ultraviolet absorbance as developed by Abbott Laboratories.

ANALYSIS

PC_{20} values were log transformed before analysis. FEV_1 and PC_{20} values were compared by analysis of variance. Paired data were analysed by the Wilcoxon sign rank test and the relation between two variables by regression analysis. Results are presented as means with the standard errors of the mean in parentheses unless otherwise stated. $p < 0.05$ was accepted as significant.

Results

SUBJECTS

Of the 11 subjects who showed a late response

Characteristics of the asthmatic subjects

Subject No	Age (y)	FEV_1 l (% pred)	PC_{20} methacholine* (mg/ml)	Drugs†
1	22	4·9 (98)	0·44	Salbutamol
2	33	3·0 (81)	0·19	Salbutamol
3	21	4·9 (117)	0·2	Salbutamol
4	22	4·1 (98)	0·26	Salbutamol
5	24	4·8 (117)	3·4	Salbutamol
6	27	4·3 (100)	0·15	Salbutamol
7	27	4·6 (105)	1·18	Salbutamol
8	23	2·6 (84)	0·57	Salbutamol
9	29	4·6 (112)	1·24	Salbutamol, beclomethasone, theophylline

*The provocative dose of inhaled methacholine needed to cause a 20% fall in baseline FEV_1.
†Inhaled, apart from the theophylline (oral) taken by subject 9.
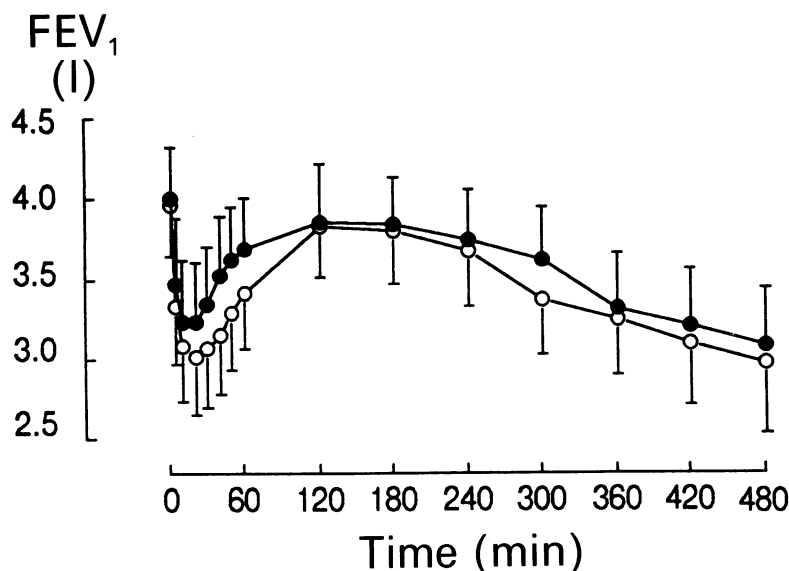
Figure 1   Effect of zileuton (800 mg orally) on early and late asthmatic responses after allergen challenge. ● Zileuton; ○ placebo. Values are means with 1 SEM.
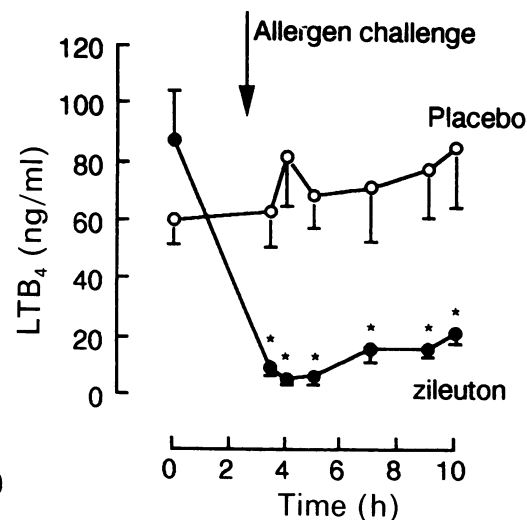

*Figure 3   Time course of effect of zileuton on calcium ionophore stimulated whole blood leukotriene (LT) B₄ production. Values are means with 1 SEM. *p < 0·005.*

to inhaled allergen, two were withdrawn. One developed an exacerbation of asthma following a chest infection after the first study day (placebo). The other subject who had an FEV₁ of just over 70% predicted was withdrawn when his FEV₁ fell to below 70% predicted before allergen challenge. The remaining nine subjects completed the study, and all results were included in the analysis. Zileuton was well tolerated and there were no important biochemical, haematological, or electrocardiographic changes after treatment.

AIRWAY RESPONSES

Baseline values before allergen challenge on placebo and treatment days were closely matched (fig 1). No period or treatment effect

was found by two way analysis according the the method of Hills and Armitage.[25] There was a trend for the fall in FEV₁ during the early asthmatic response to be less with zileuton, but this did not reach statistical significance at any point (maximum fall in FEV₁: placebo 1·08 (0·25) l, zileuton 0·83 (0·21) l; p = 0·18). The maximum difference between placebo and zileuton was 40 minutes after allergen challenge, which is after the nadir of the fall in FEV₁ (fig 1; difference in FEV₁ −0·33 (0·16) l; p = 0·08). There was no difference between placebo and zileuton in the late asthmatic response (maximum fall in FEV₁: placebo 1·16 (0·24) l, zileuton 0·92 (0·27) l; p = 0·22). There were no significant differences in the areas under the curve during the early or late phases.
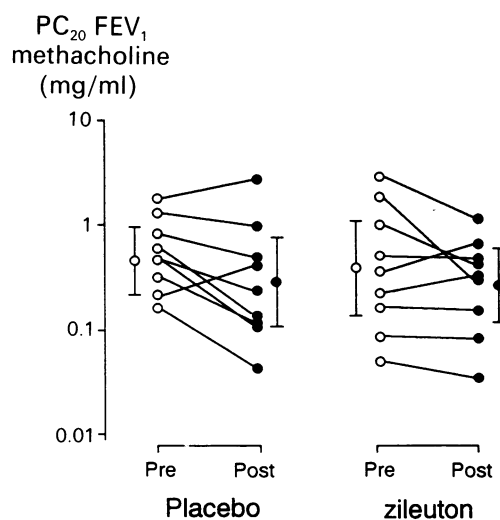

Figure 2   Effect of zileuton on airway responsiveness to inhaled methacholine before and after allergen challenge. PC₂₀ FEV₁ is the provocative dose (mg/ml) of inhaled methacholine needed to cause a fall in FEV₁ of 20% of the post-diluent value, and individual values for each subject before and after allergen challenge are shown. The geometric mean and 95% confidence interval for each day are also shown.
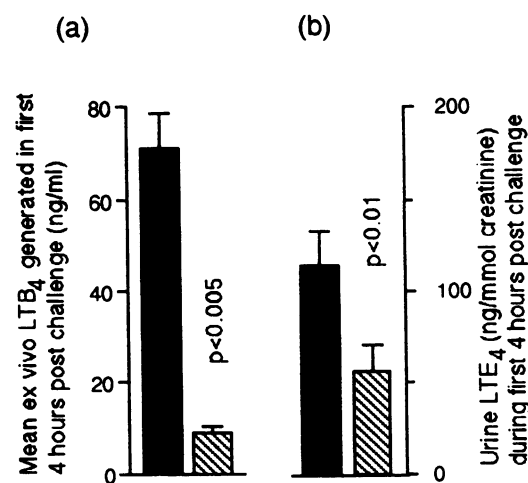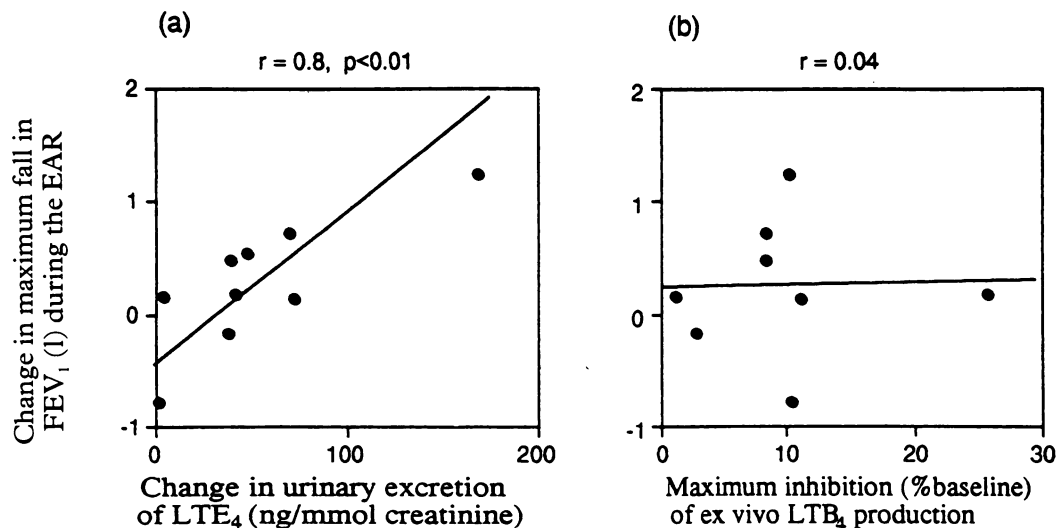

Figure 4   Effect of zileuton (▨) and placebo (■) on (a) mean ex vivo leukotriene (LT) B₄ production and (b) urine LTE₄. Mean ex vivo LTB₄ production refers to mean whole blood calcium ionophore stimulated LTB₄ production for four hours after allergen challenge. Urine LTE₄ is the total urinary excretion of LTE₄ over four hours. Values are means with 1 SEM.

Figure 5 Scatter diagrams showing the linear regression relationships between zileuton induced change in maximum fall in FEV_1 during the early asthmatic response (EAR) and reduction in urinary leukotriene (LT) E_4 production.

(a)

$r = 0.8, \ p<0.01$

Change in maximum fall in FEV_1 (l) during the EAR

Change in urinary excretion of LTE_4 (ng/mmol creatinine)

(b)

$r = 0.04$

Maximum inhibition (%baseline) of ex vivo LTB_4 production

Baseline airway responses to inhaled metacholine before allergen challenge were very similar for placebo and zileuton (geometric mean PC_{20} FEV_1 = 0·41 (95% confidence interval 0·20–0·84) and 0·44 mg/ml (0·15–0·78). The increase in airway responsiveness after allergen challenge did not differ significantly after placebo and zileuton treatment (before/after challenge PC_{20} FEV_1 metacholine: placebo 2·13, zileuton 1·89; fig 2).

EFFECTS ON IN VIVO AND EX VIVO LEUKOTRIENE GENERATION

Zileuton substantially inhibited calcium ionophore stimulated whole blood LTB_4 production with maximum inhibition (93·1% of baseline; p < 0·005) four hours post-dose; after the dose had been given significant inhibition was still present at 10 hours after the dose (63·5% of baseline; p < 0·005; fig 3). Mean urinary LTE_4 excretion after allergen challenge was reduced by about half by zileuton treatment (placebo 111·5 (23·5), zileuton 58·2 (14·8) ng/mmol creatinine; p < 0·01; fig 4).

The zileuton induced change in maximum fall in FEV_1 for the early asthmatic response correlated with the reduction in urinary LTE_4 production (r = 0·8, p < 0·01; fig 5) but not with the inhibition of calcium ionophore stimulated whole blood LTB_4 production (r = 0·04, p > 0·5).

PLASMA ZILEUTON CONCENTRATIONS

Mean plasma zileuton concentrations three and a half, four, and five hours after ingestion were 3·37 (0·36), 3·03 (0·39) and 2·6 (0·27) μg/ml respectively on the days of zileuton treatment; no drug was detected on the placebo day. There was no relation between the peak drug concentrations and change in FEV_1 during the early and late asthmatic responses, reduction in urinary LTE_4 excretion, or calcium ionophore stimulated whole blood LTB_4 production.

Discussion

We found that zileuton at the dose used in the study partially reduced urinary LTE_4 excretion

and substantially reduced calcium ionophore stimulated whole blood LTB_4 production ex vivo. There was a trend towards a reduction in the fall in FEV_1 during the early asthmatic response with zileuton, but neither this nor the late asthmatic response or increase in airway responsiveness differed significantly between zileuton and placebo. The change in the early asthmatic response induced by zileuton correlated with the change in urinary LTE_4 concentrations but not with the change in ex vivo LTB_4 production.

In vivo activation of the 5-lipoxygenase pathway in asthmatic patients during an asthmatic attack has been shown by the increased urinary excretion of LTE_4,[23] and by increased concentrations of cysteinyl leukotrienes in bronchoalveolar lavage fluid recovered by fibreoptic bronchoscopy during the early[26] and late[27] responses to inhaled allergen challenge. In the present study the activation of 5-lipoxygenase in airways was assessed by urinary excretion of LTE_4 as this is simple and non-invasive and did not interfere with measurements of lung function. Urinary LTE_4 excretion after allergen challenge is likely to reflect airway leukotriene generation, as there was no evidence of systemic effects in any subjects. Zileuton reduced the urinary excretion of LTE_4 by about half, though not down to the range found in non-asthmatic subjects in this laboratory.[23] This suggests that 5-lipoxygenase activity was only partially inhibited, and may explain the non-significant reduction in bronchoconstriction during the early asthmatic response. A significant reduction of airway response to allergen may be seen only if more complete inhibition of leukotrienes generation can be achieved, as the leukotrienes are very potent biological agents: the cysteinyl leukotrienes are thousands of times more potent than histamine in causing bronchoconstriction,[28] and LTB_4 is one of the most potent chemotactic agents known.[29]

The early bronchoconstrictor response after allergen challenge is thought to be due to release of mediators, such as histamine and leukotrienes, from inflammatory cells via IgE mediated mechanisms. Although not statis-

tically significant, the maximum reduction of the fall in FEV_1 produced by zileuton was after the nadir in the fall in FEV_1 following allergen challenge. This time course of action is consistent with the known action of leukotrienes and histamine, inhaled cysteinyl leukotrienes causing maximum bronchoconstriction at about 15 minutes,[4-6] and histamine somewhat earlier. Leukotrienes are not stored by resting cells (unlike histamine), and are generated only after stimulation, so they would be expected to contribute more to the latter part of the early bronchoconstrictor response after allergen challenge. In a study by Britton and coworkers, in which a leukotriene antagonist, L649923, reduced the early response to allergen, the maximum effect was seen after the nadir of the early asthmatic response.[11] In another study an antihistamine was effective in the first 15 minutes of the early asthmatic response after allergen challenge.[30] The significant correlation between inhibition of in vivo LTE_4 generation and reduction in early asthmatic response also supports the role of the leukotrienes as mediators in the early asthmatic response. Although the pattern of change in the early asthmatic response is similar to that in the study of Britton *et al*,[11] the lack of statistical significance in our study may be due to a type II error.

Although leukotriene production has been shown during the late asthmatic response following allergen challenge,[27] zileuton did not reduce the late fall in FEV_1 significantly in the present study. This may be because the activity of zileuton in the single dose of 800 mg used in this study may have been insufficient by that time, particularly when it was only partially effective during the early phase as measured by urinary LTE_4 levels. In two patients where serum drug concentrations seven hours after the dose were available little zileuton was detected.

Previous studies of 5-lipoxygenase inhibitors in man have depended on ex vivo methods of assessing its activity. In the present study zileuton was more effective in inhibiting ex vivo calcium ionophore stimulated whole blood LTB_4 production than in inhibiting leukotriene production in vivo as reflected by urinary LTE_4 excretion, and there was no correlation between these two variables. There are several possible explanations. The lesser effect in vivo may be due to inadequate penetration of zileuton into airway tissues, or a higher plasma concentration may be needed to suppress airways 5-lipoxygenase adequately. Alternatively, different inflammatory cells may have different susceptibilities to the effect of zileuton, such that the mast cells, macrophages, or eosinophils in the airways may not have been affected as much as neutrophils in the peripheral blood. In addition, the cellular generation of the cysteinyl leukotrienes may be less affected than that of LTB_4. The lack of relation between the changes in leukotriene generation ex vivo and the early asthmatic response suggest that this is not a useful method for assessing the clinical efficacy of 5-lipoxygenase inhibitors in man.

In this study a single oral dose (800 mg) of zileuton was only partially effective in inhibiting activation of 5-lipoxygenase in vivo by inhaled allergen despite near complete ex vivo inhibitory activity. There was a trend towards a reduction in the early asthmatic bronchoconstrictor response but this was not significant. Measurement of leukotriene production in vivo rather than ex vivo may be more useful in future studies of 5-lipoxygenase inhibitors in man. Our results would fit a role for the leukotrienes in the early asthmatic response to inhaled allergen, particularly if inhibition of leukotriene related airway responses requires near complete inhibition of the 5-lipoxygenase enzyme in vivo. Further studies with higher or repeated doses of zileuton or with more potent 5-lipoxygenase inhibitors in man are needed.

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