

Original articles

Effect of the 5-lipoxygenase inhibitor ZD2138 on allergen-induced early and late asthmatic responses

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Abstract

Background - Leukotrienes are lipid mediators generated from arachidonic acid by the 5-lipoxygenase pathway which may play an important part in the pathophysiology of asthma. Previous studies have demonstrated attenuation of the allergen-induced early and late asthmatic responses by leukotriene receptor antagonists. The effect of the 5-lipoxygenase inhibitor ZD2138, a non-redox lipoxygenase inhibitor which inhibits leukotriene synthesis for 24 hours after single doses of 350 mg, on allergen-induced early and late asthmatic responses has been assessed.

Methods - Eight asthmatic subjects with baseline FEV₁ >70% were studied. On screening, all subjects developed an allergen-induced biphasic asthmatic response to grass pollen, cat dander, or house dust mite. ZD2138 (350 mg) or placebo was given on two occasions separated by two weeks in a randomised double blind fashion. Allergen inhalation challenge was performed four hours after dosing and FEV₁ was measured for eight hours. The inhibitory activity of ZD2138 on the 5-lipoxygenase pathway was assessed by measurements of calcium ionophore-stimulated generation of LTB₄ in whole blood *ex vivo* and by analysis of urinary LTE₄ levels before administration of drug or placebo and at regular intervals after oral drug dosing and allergen challenge.

Results - ZD2138 produced no significant bronchodilatation or attenuation of the early or late asthmatic response, although there was 82% inhibition of whole blood generation of LTB₄ in response to calcium ionophore stimulation and 52% reduction in urinary excretion of LTE₄.

Conclusions - In asthmatic subjects the 5-lipoxygenase inhibitor ZD2138 did not protect against allergen-induced asthmatic responses, despite substantial inhibition of 5-lipoxygenase.

Leukotrienes are lipid mediators derived from the oxidative metabolism of arachidonic acid by the 5-lipoxygenase pathway. LTB₄ has potent chemoattractant properties for granulocytes and monocytes,¹⁻⁴ enhances complement receptor expression,^{2,3} increases neutrophil adherence to endothelium,⁵ and causes neutrophil aggregation and degranulation.^{3,6} The cysteinyl leukotrienes LTC₄, LTD₄, and LTE₄ are potent bronchoconstrictors,⁷ stimulate bronchial mucus secretion,^{8,9} increase venopermeability,^{10,11} and increase airways hyperresponsiveness of asthmatic subjects.^{12,13} Leukotrienes have been recovered from asthmatic airways by bronchoalveolar lavage¹⁴⁻¹⁷ and increased levels of LTE₄ have also been detected in the urine of asthmatic subjects during an acute asthma attack^{18,19} and following allergen challenge.^{18,20-22} Both early and late asthmatic responses and the increased airway hyperresponsiveness provoked by allergen challenge are attenuated by administration of cysteinyl leukotriene receptor antagonists.²³⁻²⁶ An alternative way to inhibit the action of leukotrienes is to inhibit their biosynthesis. We have therefore evaluated the effect of a novel 5-lipoxygenase inhibitor, ZD2138 (previously reported as ICI D2138), on allergen-induced asthma. ZD2138 is a non-redox lipoxygenase inhibitor²⁷ which is well tolerated and, at single oral doses of 350 mg and above, inhibits leukotriene synthesis for 24 hours.²⁸

Methods**SUBJECTS**

Eight non-smoking atopic men with asthma of mean age 27 (range 23-37) years were studied (table). Their mean (SE) resting forced expiratory volume in one second (FEV₁) was 4.02 (0.25) l (mean predicted 97%; range 74-124%). One subject took no medication, one took regular inhaled beclomethasone dipropionate (200 µg daily), salmeterol (200 µg daily) and salbutamol as required, and six subjects took salbutamol as required. All subjects had positive immediate skin weal reactions to either cat dander, *Dermatophagoides pteronyssinus*, or grass pollen (*Phleum pratense*). The study was approved by the Guy's Hospital ethics committee and written informed consent was obtained from each subject.

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Age, baseline FEV₁ values, medication, and allergen used for bronchial provocation in the eight subjects

Subject	Age (years)	FEV ₁ (litres)	% predicted FEV ₁	Drugs	Allergen
1	26	3.29	74	S	GP
2	25	4.96	105	S, B, SM	DP
3	37	4.11	98	—	GP
4	24	3.98	102	S	Cat
5	26	3.43	93	S	DP
6	27	5.2	124	S	GP
7	30	3.67	92	S	DP
8	23	3.51	88	S	Cat

S = salbutamol; B = beclomethasone dipropionate; SM = salmeterol; GP = grass pollen; DP = *Der-matophagoides pteronyssinus*; Cat = cat dander.

STUDY PROTOCOL

Subjects were studied on three days, each separated by two weeks. On day 1 a medical history was taken and the subjects underwent general physical examination with an ECG. Blood was taken for a full blood count, clotting studies, estimation of electrolytes and urea, and liver function tests. Urine was examined by microscopy and tested for glucose, protein, and blood. Skin testing and inhaled allergen challenge were then performed. Subjects with a 20% or greater fall in FEV₁ within 15 minutes of allergen inhalation (early response) and at 4–8 hours after allergen challenge (late response) continued with study days 2 and 3. Salmeterol was discontinued for 48 hours, inhaled corticosteroids for 24 hours, and salbutamol for at least eight hours before each study day until 24 hours after allergen challenge.

On days 2 and 3 350 mg ZD2138 (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) or matching placebo was given after an overnight fast in a randomised, double blind fashion. Four hours later allergen challenge was performed as on day 1. Continuous ambulatory ECG was monitored on days 2 and 3 for 24 hours. Urine was collected before drug or placebo administration and then immediately before and three, six, and nine hours after allergen challenge. The patients did not pass urine between the specified collection times. Blood for determination of ex vivo LTB₄ generation was taken before and four (pre-allergen), 12, and 24 hours after drug administration. Days 2 and 3 were separated by two weeks.

SKIN TESTING

Subjects were prick tested to cat dander, *Der-matophagoides pteronyssinus*, and grass pollen (ALK, Horsholm, Denmark) at a concentration of 81920 square units/ml of Coca's solution (4% phenol, 2.75% sodium bicarbonate, and 5% sodium chloride (w:v)). The allergen producing the largest weal diameter was chosen for a dose ranging study of 12 doubling dilutions in Coca's solution. One doubling dilution less than that producing a skin weal of 2 mm greater than diluent alone was chosen as the starting dilution for bronchial provocation.

SPIROMETRY AND ALLERGEN CHALLENGE

Two recordings of FEV₁ were made with a dry wedge bellows spirometer (Vitalograph Ltd, Buckingham, UK) at each time point and the

highest value was used. Further readings were only made if the first two values were not within 10% or were technically inadequate. Nebulised solutions of Coca's solution and allergen were administered at room temperature using a Hudson nebuliser (Henleys Medical Supplies, Middlesex, UK) linked to a breath actuated dosimeter. Delivery of air to the nebuliser was regulated to a pressure of 138 kPa (20 psi) for a duration of 0.6 seconds from the start of each breath. There was no time lag between inspiration and actuation of the dosimeter. Under these conditions the nebuliser delivers droplets with a mass median aerodynamic diameter of 1.6 µm and the output of the nebuliser is 2.6 µl per actuation. After baseline measurements of FEV₁ the subjects inhaled five breaths of Coca's solution as control. Each dose consisted of five submaximal breaths starting at functional residual capacity followed by a five second breath hold. If the decrease in FEV₁ following Coca's solution was <10% the subjects underwent allergen challenge. Incremental doubling concentrations of allergen diluted in Coca's solution were used for bronchial challenge at 15 minute intervals with FEV₁ measurements at 5, 10, and 15 minutes until a fall in FEV₁ of at least 20% was achieved. Further measurements of FEV₁ were made at 20, 30, and 60 minutes and then hourly until eight hours after allergen challenge. Subsequent allergen challenges on days 2 and 3 were performed using the final three allergen concentrations leading to a 20% fall in FEV₁ and were given sequentially at 10 minute intervals. The coefficient of repeatability of the early and late asthmatic reactions following allergen challenge in 26 subjects in our laboratory is 38% and 27% respectively. All eight subjects were given 200 µg salbutamol routinely at the end of the period of urine collection. No subject required treatment.

MEASUREMENT OF URINARY LTE₄

The free radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidinoxy free radical (4-hydroxy-TEMPO; Sigma Chemical Company, Poole, Dorset, UK) was added to each urine sample immediately after collection to a final concentration of 1 mmol/l, the samples were adjusted to pH 9 with NaOH to stabilise endogenous leukotriene metabolites and stored at -70°C until analysed. Urinary LTE₄ levels were measured as described previously.²⁹ The stored urine samples were thawed and 50 µl of a solution containing [³H] LTE₄ (128.5 Ci/mmol; NEN, DuPont, Boston, USA) was added to 10 ml urine to give 4000 disintegrations per minute as an internal standard. After adding 250 µl glacial acetic acid to bring the pH to 3.5–3.8 the 10 ml sample was loaded onto a 10 µm precolumn (Ultrasil ODS, 3.4 mm × 4.5 cm, Beckman, USA) to wash off polar metabolites with a phosphate buffer (0.1% NaH₂PO₄, pH 3.8) for eight minutes and then for 12 minutes with methanol:phosphate buffer in the proportions 50:50 (v:v). The sample was then retrogradely eluted onto a reversed phase analytical column (Ultrasil ODS 4.5 mm × 25 cm, Altex) that had been equilibrated in 62%

methanol:37.8% water:0.1% acetic acid:0.1% EDTA (v:v:v:w) at pH 5.6 at a flow rate of 1 ml/min. One minute fractions were collected and the fraction containing peak radioactivity corresponding to the internal standard was identified by counting 250 μ l of the eluent in a β liquid scintillation counter (Tricarb 1900CA, Packard Instrument Co, USA). The remaining 750 μ l of those fractions containing [3 H] LTE₄ and the two fractions eluted before and after the peak were dried under vacuum and resuspended in 250 μ l of 10 mmol/l Tris HCl buffer (pH 7.4). The concentration of immunoreactive LTE₄ was assessed by radioimmunoassay as described previously.^{29,30}

Samples to be measured or defined dilutions of standard LTE₄ and rabbit anti-LTC₄ (kind gift of Dr A Ford Hutchison, Merck Frosst, Dorval, Quebec, Canada) were incubated with labelled [3 H] LTE₄ (128.5 Ci/mmol, NEN, Dupont, Boston, USA) in a total volume of 300 μ l Tris HCl buffer pH 7.4 for one hour at room temperature. 200 μ l of 1% charcoal/dextran was added to each tube to absorb unbound LTC₄ and the tubes centrifuged at 2000 *g* for 15 minutes at 4°C. The radioactivity of 400 μ l of the supernatant was counted in a liquid scintillation counter. The quantity of immunoreactive LTE₄ was interpolated from a standard curve which was linear in the range 0.01–0.03 ng LTE₄. The LTC₄ antibody binds to LTC₄, LTD₄, and LTE₄ with 50% binding at 0.03 ng/ml, 0.06 ng/ml, and 0.09 ng/ml respectively.

Urinary creatinine concentrations were measured (Mira Analyser, Hoffman Roche, Welwyn) by the kinetic picrate method without deproteinisation.³¹ LTE₄ concentrations were expressed as pg/mg creatinine and were calculated from measured immunoreactivity minus background, corrected for recovery and dilution.

cium ionophore-stimulated ex vivo LTB₄ production in whole blood. Blood was collected into heparinised tubes and calcium ionophore (A23187) was added to 500 μ l whole blood in triplicate to a final concentration of 2.5 μ mol/l. The blood was incubated for 20 minutes at 37°C, centrifuged at 3600 rpm for one minute, and the supernatant stored at –20°C. LTB₄ was assayed subsequently by radioimmunoassay as previously described.³²

The rabbit anti-LTB₄ antibody used for radioimmunoassay only crossreacts significantly with 6-*trans* LTB₄ (60%), 20-OH LTB₄ (41%), and 12(R,S)HETE (33%). Scatchard analysis showed this antibody to be of high affinity for LTB₄ ($K_a = 4.9 \times 10^{-11}$ mol/l).

DATA ANALYSIS

Comparison of urinary LTE₄ and whole blood LTB₄ levels after ZD2138 and placebo were analysed non-parametrically using the Wilcoxon signed rank test on the individual areas under the concentration–time curves. Airway responses to allergen and the differential effect of placebo and ZD2138 were assessed by calculation of the area under the FEV₁–time curves for the following periods: 0–4 hours after drug administration to assess bronchodilator effect; 0–1 hour after allergen to assess the early asthmatic response; and 3–8 hours after allergen to assess the late asthmatic response. Comparisons between drug and placebo days were analysed using the Wilcoxon signed rank test of the area under the FEV₁–time curves. Linear regression analysis was used to compare the percentage inhibition of urinary LTE₄ and whole blood ex vivo LTB₄ levels with both the early and late asthmatic responses. A *p* value of <0.05 was considered significant. Data are expressed as mean (SE).

MEASUREMENT OF EX VIVO LTB₄

The ability of ZD2138 to inhibit 5-lipoxygenase was also assessed by measurement of cal-

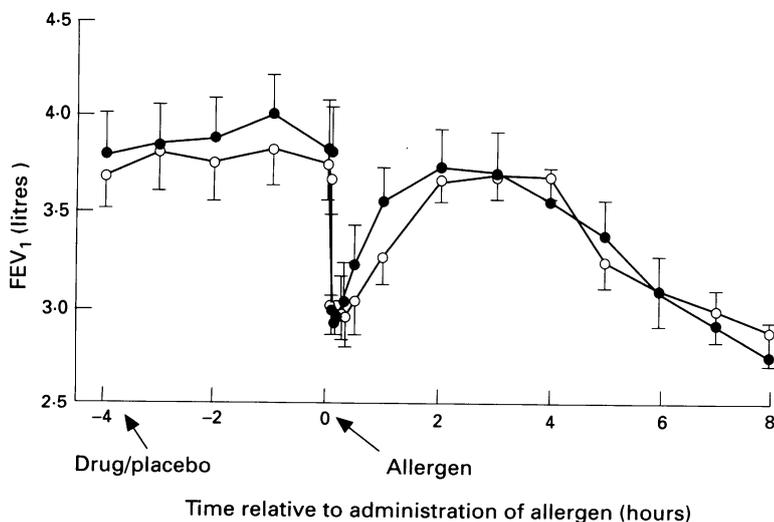


Figure 1 Time course of changes in FEV₁ following treatment with ZD2138 (●) and placebo (○) before and after allergen challenge on each study day. Study medication was administered four hours before allergen challenge (–4 hours) immediately after the FEV₁ measurement. Allergen challenge was performed at 0 time and FEV₁ was measured for eight hours afterwards. Each point denotes mean (SE) of eight subjects.

Results

AIRWAY RESPONSES

There was no significant difference in baseline FEV₁ before drug administration between the two study days. Figure 1 shows the mean change in FEV₁ after administration of placebo or ZD2138 on study days 2 and 3. There was no significant bronchodilatation during the four hours after administration of ZD2138. The FEV₁ values immediately before allergen challenge were 3.81 (0.24) l after ZD2138 and 3.68 (0.19) l after placebo and were not significantly different. Although the FEV₁–time curve during the early asthmatic response appeared to return to baseline sooner after ZD2138, there was no significant difference in the extent of the early or late asthmatic responses following allergen challenge between administration of ZD2138 and placebo as measured by the areas under the FEV₁–time curves.

INHIBITION OF EX VIVO GENERATION OF LTB₄

Figure 2 shows the mean ionophore-induced generation of LTB₄ from whole blood after placebo and ZD2138. No difference between the two baseline LTB₄ levels was found. There

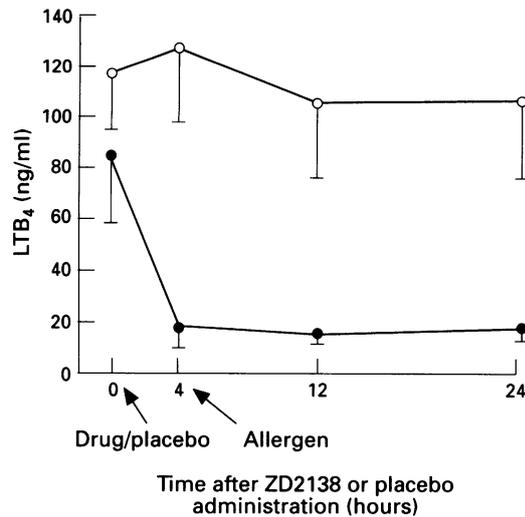


Figure 2 Whole blood ex vivo generation of LTB₄ in response to 2.5 μ mol/l A23187 before and after receiving ZD2138 (●) or placebo (○). Allergen was administered four hours after dosing. Each point denotes mean (SE) of eight subjects.

was a statistically significant difference between the ZD2138 and placebo days for the area under the curves ($p=0.01$), with an 82% reduction in LTB₄ generation on the ZD2138 day compared with placebo. Linear regression analysis demonstrated no significant correlation between percentage inhibition of ex vivo generation of whole blood LTB₄ and the early ($r=0.55$; $p=0.15$) or late ($r=-0.12$; $p=0.77$) asthmatic response.

URINARY LTE₄

There was no significant difference between baseline urinary LTE₄ levels on the two days (fig 3). Comparison of the area under the curves indicated a 52% reduction in urinary LTE₄ excretion following ZD2138 compared with that observed after administration of placebo ($p=0.013$). Linear regression analysis demonstrated no correlation between percentage inhibition of urinary LTE₄ excretion and either the early ($r=0.53$; $p=0.18$) or the late ($r=-0.23$; $p=0.58$) asthmatic response.

ZD2138 was well tolerated and no adverse events attributed to the drug occurred in this study. All eight subjects completed the study.

Discussion

There is increasing evidence that leukotrienes may contribute to the inflammation of the airways, hyperresponsiveness of the airways, and the acute bronchospasm that characterise bronchial asthma. In support of this hypothesis leukotrienes have been recovered from the bronchoalveolar lavage fluid of asthmatic subjects at rest^{14,15} and following challenge with allergen^{16,17} and isocapnic hyperventilation.³³ Increased urinary excretion of LTE₄ has been demonstrated during acute severe asthma^{18,19} and following allergen-induced asthma.^{18,20-22} If leukotrienes are important in asthma, then drugs which inhibit their generation or antagonise their action should inhibit asthmatic responses.

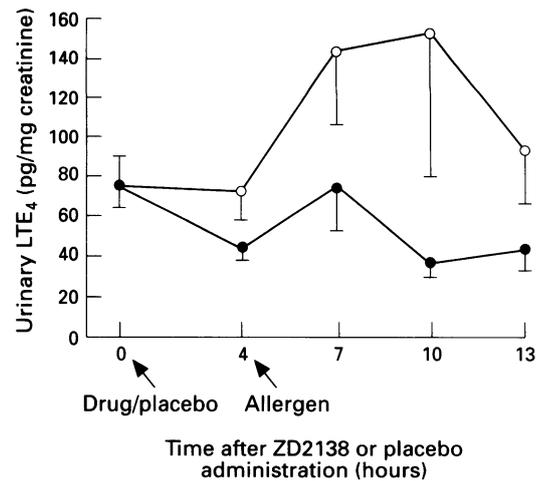


Figure 3 Urinary LTE₄ levels before and after receiving ZD2138 (●) or placebo (○). Allergen was administered four hours after dosing. Each point denotes mean (SE) of eight subjects except at 13 hours where $n=6$.

In this study we evaluated the effect of a 5-lipoxygenase inhibitor on allergen-induced asthma. ZD2138 was developed from a class of 5-lipoxygenase inhibitors that inhibit the enzyme via an enantioselective mechanism.³⁴ In vitro data on whole blood show that it is the most potent and most selective 5-lipoxygenase inhibitor yet reported,²⁷ and has a half life after absorption of 12 hours. Inhibition of 5-lipoxygenase, which is both maximal (>90%) and sustained for 24 hours, is obtained at a plasma concentration of 80 ng/ml which is achieved with a dose of 350 mg or more. Subjects were fasted in the present study to standardise oral bioavailability. 5-lipoxygenase activity was monitored by measuring whole blood generation of LTB₄ in response to calcium ionophore, and by measuring urinary excretion of LTE₄ following allergen challenge. Administration of ZD2138 led to an 86% inhibition in whole blood generation of LTB₄ at four hours after dosing immediately before allergen challenge, 85% inhibition of whole blood LTB₄ generation 12 hours after dosing during the late asthmatic response, and 83% inhibition at 24 hours after dosing. Administration of ZD2138 led to a significant decrease in urinary excretion of LTE₄ before allergen challenge and attenuated the allergen-induced rise in urinary LTE₄ excretion which was observed on the placebo day. Despite this evidence for effective inhibition of 5-lipoxygenase activity there was no attenuation of early or late asthmatic responses to allergen following administration of ZD2138. These findings are in agreement with those of Hul *et al* who found no effect of the 5-lipoxygenase inhibitor Zileuton on allergen-induced asthma,³⁵ and are in contrast to the efficacy of leukotriene receptor antagonists in allergen-induced asthma.²³⁻²⁶

There are several possible explanations for the lack of efficacy of 5-lipoxygenase inhibitors in allergen-induced asthma. Firstly, it is possible that leukotrienes do not contribute significantly to allergen-induced asthmatic reactions. This possibility seems unlikely since leukotrienes are released at the time of allergen

challenge¹⁶⁻²¹ and the more potent LTD₄ receptor antagonists attenuate allergen-induced asthmatic responses.²³⁻²⁶ Secondly, there may have been insufficient attenuation of leukotriene generation within the airway. This possibility cannot be excluded although the administration of ZD2138 inhibited ionophore-induced generation of LTB₄ in whole blood and attenuated the allergen-induced rise in LTE₄ excretion in the urine. Nevertheless it should be emphasised that neither of these assays document pulmonary release of leukotrienes alone. A third possibility is that 5-lipoxygenase inhibition in the lung diverted allergen-induced arachidonic acid metabolism to the generation of bronchoconstrictor prostaglandins via the cyclooxygenase pathway. Finally, it is possible that 5-lipoxygenase inhibition attenuates the generation not only of leukotrienes, but also of double lipoxygenase products such as the lipoxins.³⁶ Lipoxins are formed by the combined activity of 5-lipoxygenase and 15-lipoxygenase. Lipoxin A₄ (LXA₄), the major isomer, inhibits guinea pig pulmonary responses to LTC₄ in vitro³⁶ and inhalation of LXA₄ in asthmatic subjects inhibited the bronchoconstrictor response to LTC₄.³⁷ Inhibition of 5-lipoxygenase may lead to decreased generation of LXA₄ resulting in a loss of its protective effect against residual leukotrienes within the airway. This could explain the absence of the protective effect of ZD2138 in allergen challenge and would also account for the difference in efficacy of 5-lipoxygenase inhibitors and sulphidopeptide leukotriene receptor antagonists in preventing allergen-induced asthmatic reactions. Lipoxin generation was not measured in this study and so this hypothesis was not tested.

Although 5-lipoxygenase inhibition is ineffective in attenuating allergen-induced asthma, it is known that the 5-lipoxygenase inhibitor Zileuton (A64077) inhibited the airways response to cold air.³⁸ More importantly, Zileuton has also been shown to improve the severity of day to day asthma.³⁹ This inevitably raises the question of the value of allergen-induced asthma as a screen for the efficacy of certain classes of 5-lipoxygenase inhibitors. The dichotomy between efficacy in chronic asthma and in allergen-induced bronchoconstriction was also seen with dietary fish oil supplements which attenuated the late asthmatic response to allergen but had no effect on the severity of day to day asthma.^{40,41} It is interesting that another class of 5-lipoxygenase inhibitors, namely the FLAP inhibitor MK-886, attenuated both the early and late asthmatic responses.⁴² It remains to be established whether different classes of 5-lipoxygenase inhibitors and leukotriene receptor antagonists vary in their clinical profiles.

In summary, despite effective inhibition of 5-lipoxygenase, ZD2138 had no significant effect on early and late responses to allergen. This is in contrast to the efficacy of cysteinyl leukotriene receptor antagonists in allergen-induced asthma. Since there is evidence to suggest that 5-lipoxygenase inhibition is efficacious in the management of bronchial asthma, the value of allergen-induced asthma as a model in which to

predict the efficacy of this class of novel agents is questioned.

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