

Selective Inducible Nitric Oxide Synthase Inhibition Has No Effect on Allergen Challenge in Asthma

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Rationale: Exhaled breath nitric oxide (F_{ENO}) is increased in asthma. NO is produced predominantly by inducible nitric oxide synthase (iNOS).

Objectives: We evaluated the selective and potent iNOS inhibitor GW274150 in asthma.

Methods: Twenty-eight steroid-naïve patients with asthma participated in a double-blind, randomized, double-dummy, placebo-controlled, three-period cross-over study. Subjects received GW274150 (90 mg), montelukast (10 mg), or placebo once daily for 14 days. F_{ENO} was assessed predose on Days 1, 7, 10, and 14. Adenosine 5'-monophosphate (AMP) challenge was performed on Day 10, allergen challenge on Day 14 followed by methacholine challenge (MCh) 24 hours later, and then bronchoscopy.

Measurements and Main Results: GW274150 reduced predose F_{ENO} by 73, 75, and 71% on Days 7, 10, and 14, respectively, compared with placebo. Montelukast did not reduce F_{ENO}. GW274150 did not inhibit AMP reactivity whereas for montelukast there was a trend toward inhibition: the mean doubling dose difference versus placebo was 0.64 (95% confidence interval [95% CI], 0 to 1.28). GW274150 did not inhibit early (EAR) and late (LAR) asthmatic responses to allergen, or MCh reactivity, despite reduced F_{ENO} levels. Montelukast inhibited EAR and LAR FEV₁; the mean difference versus placebo for minimal FEV₁ was 0.37 L (95% CI, 0.19 to 0.55) and 0.18 L (95% CI, 0.04 to 0.32), respectively. MCh reactivity was inhibited by montelukast (mean doubling dose difference vs. placebo, 0.51; 95% CI, 0.02 to 1.01). GW274150 also had no effect on inflammatory cell numbers in bronchoalveolar lavage fluid after allergen challenge.

Conclusions: Selective iNOS inhibition effectively reduces F_{ENO} but does not affect airway hyperreactivity or airway inflammatory cell numbers after allergen challenge in subjects with asthma.

Clinical trial registered with www.clinicaltrials.gov (NCT00273013).

Keywords: nitric oxide; bronchial hyperreactivity

Levels of nitric oxide in exhaled air (F_{ENO}) are significantly elevated in patients with asthma compared with healthy volunteers (1–3). Nitric oxide (NO) is formed from L-arginine, O₂, and NADPH by the catalytic activity of nitric oxide synthase (NOS) enzymes (4, 5). Neuronal NOS (nNOS or NOSI, NOS1) and endothelial NOS (eNOS or NOSIII, NOS3) are constitutive enzyme isoforms. Inducible NOS (iNOS or NOSII, NOS2)

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Exhaled nitric oxide (NO) is produced by inducible nitric oxide synthase (iNOS), and levels are high in asthma. iNOS is therefore a potential therapeutic target in asthma.

What This Study Adds to the Field

Administration of a selective iNOS inhibitor to patients with asthma reduced NO levels, but had no effect on allergen responsiveness. Targeting NO may not be an effective intervention in asthma.

expression is increased in airway epithelial and inflammatory cells from subjects with asthma compared with healthy control subjects, suggesting a key role for this enzyme in NO overproduction in patients with asthma (6–8).

There is much debate as to whether NO is a harmful or beneficial molecule in the airways (9–11). The effects of NO are cell type and concentration specific, and highly dependent on the presence of other factors, such as reactive oxygen species, in the local microenvironment. The harmful effects of NO include increased nitrosative stress through the production of harmful reactive nitrogen species such as peroxynitrite, which can modify tyrosine in proteins to form 3-nitrotyrosine (3-NT) (6, 7, 10, 11). In contrast, NO and related NO species may also act as bronchodilators and have antiinflammatory effects (11–13).

Inhibitors of the constitutive NOS isoforms produce a range of undesirable effects including hypertension (14). In addition, the nonselective NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) did not inhibit responses to allergen challenge in patients with asthma (15). A rise in adenosine 5'-monophosphate (AMP) and histamine reactivity with L-NAME has also been observed, perhaps because of inhibition of broncho-protective NO produced by constitutive NOS (16). GW274150 [(S)-2-amino-7-acetamidino-5-thioheptanoic acid] is a long-acting inhibitor of iNOS that has a high degree of selectivity for iNOS versus both constitutive NOS isoforms, eNOS (more than 100-fold) and nNOS (more than 80-fold) (17, 18). In animal models of pulmonary inflammation, GW274150 protects against the late airway response to an acute allergen challenge (19, 20). GW274150 has also shown protective effects in a range of rodent models of inflammation outside the lung (21–23). GW274150 (24) and the selective iNOS inhibitor SC-51 [the prodrug of L-NIL, N⁶-(1-iminoethyl)-L-lysine 5-tetrazole amide] (25) substantially reduce F_{ENO} levels in patients with mild asthma. We hypothesized that GW274150 would also effectively treat pulmonary inflammation in patients with asthma.

The main aim of this study was to investigate whether therapeutic benefit could be achieved by highly selective inhibition of iNOS with GW274150 in the experimental allergen

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challenge model of allergic asthma. We performed a double-blind, placebo-controlled, three-way cross-over study in steroid-naïve patients with asthma to compare the effects of GW274150, montelukast, and placebo on FE_{NO} levels, airway hyperreactivity, and inflammatory cells. Some of the results of this study have already been presented in abstract form (26).

METHODS

Subjects

Twenty-eight steroid-naïve patients with physician-diagnosed asthma for at least 6 months were recruited. Subjects were required to be aged 18 to 55 years and nonsmokers for at least 6 months with less than a 10-pack-year history. At screening patients were required to have an FEV_1 greater than 65% predicted, have a positive skin test to either house dust mite, grass pollen, or cat allergen, and demonstrate both an early and late asthmatic reaction to one of these allergens when inhaled. They were also required to have AMP and methacholine PC_{20} (provocative concentration causing a 20% fall in FEV_1) less than 100 and 8 mg/ml (or $PD_{20} < 3.2$ mg), respectively. See the online supplement for a list of the exclusion criteria. All patients provided written, informed consent. The study was approved by the local research ethics committee.

Study Design

This was a two-center, double-blind, randomized, double-dummy, placebo-controlled, three-period cross-over study. Figure 1 shows the study design. Eligible subjects received GW274150 (90 mg), montelukast (Singulair, 10 mg; Merck, Whitehouse Station, NJ), and placebo once daily for 14 days in random order. During each treatment period subjects were instructed to take the study medication at the same time of day. The washout period was 14–28 days between treatment periods to allow sufficient time between allergen challenges and flexibility of study visit scheduling. Subjects were required to fast for 8 hours before observed dosing on Days 1, 7, 10, and 14 and to refrain from short-acting β -agonist use for 8 hours before each clinic visit. Heart rate, blood pressure, ECG, and FEV_1 were measured on Days 1, 7, 10, and 14. FE_{NO} was performed immediately predose on Day 1 (referred to as “baseline measurements”) and on Days 7, 10, and 14. On Day 10, subjects underwent an AMP challenge within 6 hours of dosing. On Day 14, an inhaled allergen challenge was performed 1 hour postdose and FE_{NO} was measured 2, 6, 9, and 24 hours postdose. A methacholine challenge was then performed on Day 15, 23 hours after allergen challenge (24 hours postdose). A bronchoscopy (see the online supplement for details) to obtain bronchial biopsies and bronchoalveolar lavage (BAL) was performed after recovery from the methacholine challenge on Day 15. All subjects underwent a bronchoscopy during the placebo treatment period and were randomized to undergo bronchoscopy after treatment with either GW274150 or montelukast. Adverse events and β -agonist use were monitored throughout the study with the aid of diary cards.

Allergen, AMP, and Methacholine Challenges

See the online supplement for a description of the bronchial challenges performed.

FE_{NO}

FE_{NO} was measured with a CLD 88 analyzer (ECO MEDICS, Duernten, Switzerland) at a flow of 50 ml/second as described in the online supplement.

Immunohistochemistry

The immunohistochemical methods used to stain bronchial biopsies for iNOS and 3-NT expression are described in the online supplement, as is the methodology for BAL centrifuged cell preparation (Shandon Cytospin; Thermo Fisher Scientific, Waltham, MA) and differential cell counting. iNOS and 3-NT expression in the epithelium and subepithelium were expressed as the mean intensity of positive staining, and positive staining was expressed as a percentage of the total tissue area.

Statistical Analysis

Bronchial challenge end points were analyzed using mixed effect models with treatment and period fitted as fixed effects and subject fitted as a random effect. Confidence intervals (CIs) where quoted are 95% CIs. See the online supplement for further statistical details.

RESULTS

Twenty-eight subjects (see Table 1 for demography) were randomized, of whom 25 completed the study (26 received GW274150, 25 montelukast, and 28 placebo). The three withdrawals were due to worsening asthma (during treatment with placebo), a vasovagal episode during intravenous cannulation, and for personal reasons. The numbers of patients screened, excluded, randomized, and withdrawn are summarized in Figure 2.

Study medications were well tolerated. The most frequently reported adverse events were headache (reported by 7 subjects [27%] taking GW274150, 6 [24%] taking montelukast, and 8 [29%] taking placebo) and pharyngolaryngeal pain (reported by 3 [12%] subjects taking GW274150, 3 [12%] taking montelukast, and 3 [11%] taking placebo). No treatment-related trends were observed in safety laboratory parameters, blood pressure, ECG parameters, β -agonist reliever use, and predose FEV_1 values (Table 2).

Exhaled NO

Geometric mean and 95% confidence intervals for predose FE_{NO} concentrations on Days 1, 7, 10, and 14, and postdose concentrations on Day 14, are summarized in Figure 3 (and see Table E1 in the online supplement). GW274150 reduced predose FE_{NO} measurements by 73% (95% CI, 64 to 79%), 75% (95% CI, 67 to 80%), and 71% (95% CI, 64 to 78%) on Days 7, 10, and 14, respectively, at the trough of the effect, compared with placebo. In contrast, there was no evidence of a statistically significant difference in FE_{NO} measurements during treatment with montelukast compared with placebo.

Allergen Challenge

Early asthmatic response. The early asthmatic response (EAR) FEV_1 change on Day 14 was similar during treatment with GW274150 and placebo (Figure 4). There was no significant difference for either the minimal FEV_1 or weighted mean FEV_1 (see Table 3). In contrast, montelukast inhibited the EAR compared with placebo, with on average a 42 and 62% attenuation of the minimal FEV_1 and weighted mean FEV_1 , respectively.

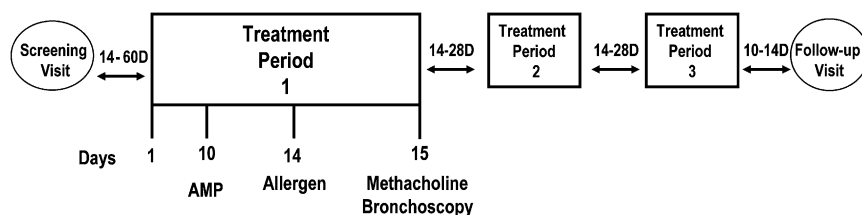


Figure 1. Study design flow chart. Bronchial challenges and bronchoscopy days are shown for treatment period 1, and were identical for periods 2 and 3. Methacholine challenge was performed before bronchoscopy on Day 15. Exhaled breath nitric oxide (FE_{NO}) and FEV_1 were measured predose on Days 1, 7, 10, and 14 in each treatment period. FE_{NO} and FEV_1 were measured after allergen challenge as described in METHODS. AMP = adenosine 5'-monophosphate; D = day.

TABLE 1. SUBJECT DEMOGRAPHY

Variable	Value
Age, yr	30.6 (21–43)
Sex	17 male/11 female
FEV ₁ , L	3.35 (2.14–4.60)
FEV ₁ , % predicted	96.7 (65.8–128.4)
F _{ENO} , ppb	37.7 (7.8–102.2)
Allergens used for bronchial challenge	18 dust mite, 6 cat dander, 4 grass mix
Methacholine PC ₂₀ , mg/ml*	0.56 (0.07–7.01)
AMP PC ₂₀ , mg/ml†	9.43 (0.39–320)

Definition of abbreviations: AMP = adenosine 5'-monophosphate; F_{ENO} = exhaled breath nitric oxide; PC₂₀ = provocative concentration causing a 20% fall in FEV₁.

Data were collected before the first treatment period. Values represent mean (range), except for PC₂₀ and F_{ENO} data, which are presented as geometric mean (range).

* Twenty-three of 28 subjects.

† Twenty-seven of 28 subjects.

Late asthmatic response. The LAR change from postsaline FEV₁ from 4 to 10 hours postallergen on Day 14 was similar during treatment with GW274150 and placebo (Figure 2). There was no significant difference for either the minimal FEV₁ or weighted mean FEV₁ (see Table 3). In contrast, montelukast inhibited the LAR compared with placebo, with 17 and 26% attenuation of the minimal FEV₁ and weighted mean FEV₁, respectively.

F_{ENO} post-allergen challenge. F_{ENO} concentrations compared with baseline (predose on Day 1) were significantly reduced by GW274150 compared with placebo post-allergen challenge; at 2 hours postdose (1 h after allergen challenge) the reduction was on average 89% (95% CI, 86 to 92%) (see Figure 1; and see the online supplement for a full listing of F_{ENO} data). At 23 hours post-allergen challenge, F_{ENO} levels were increased in all groups compared with the Day 14 predose levels (Figure 1, and see the online supplement for a full listing of F_{ENO} data). The increase was similar in the placebo and montelukast groups: an approximately 1.6-fold increase. There was a 1.8-fold increase in the GW274150 group. However, the absolute F_{ENO} concentrations in the GW274150 group were still considerably lower compared with the placebo and montelukast groups.



Figure 2. Flow of patients through study, showing number screened, randomized, withdrawn, and completed. Excluded patients did not fulfill the inclusion criteria.

TABLE 2. MEAN FEV₁ PREDOSE VALUES ON DAYS 1, 7, 10, AND 14

Day	Placebo	GW274150	Montelukast
1	3.15 (2.85, 3.45)	3.25 (2.97, 3.53)	3.28 (2.97, 3.59)
7	3.19 (2.87, 3.50)	3.26 (2.96, 3.55)	3.32 (3.03, 3.62)
10*	3.24 (2.95, 3.53)	3.32 (3.01, 3.62)	3.36 (3.08, 3.64)
14	3.25 (2.95, 3.55)	3.22 (2.92, 3.51)	3.21 (2.93, 3.48)

Values represent means and 95% confidence intervals.

* Day 10 values are pre-AMP challenge.

Methacholine challenge. Adjusted geometric mean methacholine PC₂₀ (mg/ml) values, determined 23 hours after the allergen challenge, were 0.32 (95% CI, 0.22 to 0.48), 0.28 (95% CI, 0.19 to 0.42), and 0.46 (95% CI, 0.31 to 0.69) after treatment with placebo, GW274150, and montelukast, respectively. There was no difference between the methacholine reactivity during dosing with GW274150 (90 mg) and placebo (mean doubling dose difference, −0.19; 95% CI, −0.69 to 0.30). In contrast, methacholine reactivity was inhibited by montelukast compared with placebo (mean doubling dose difference, 0.51; 95% CI, 0.02 to 1.01).

Bronchoscopy samples. The epithelial intensity of the expression of iNOS and 3-NT 24 hours after allergen challenge was not different between placebo and active treatment groups (see Table 4). The percentage area of epithelium expressing iNOS and 3-NT was not reduced by the active treatments compared with placebo, and a high degree of variability in the data was observed. See the online supplement for examples of iNOS and 3-NT staining. The BAL cell counts were similar in the three treatment arms (see Table 5); neither GW274150 nor montelukast decreased inflammatory cell numbers.

AMP Challenge

The adjusted geometric mean AMP PC₂₀ (mg/ml) at 6 hours postdose on Day 10 were 5.2 (95% CI, 2.5 to 11.1), 6.4 (95% CI, 3.0 to 13.6), and 8.2 (95% CI, 3.8 to 17.4) after treatment with placebo, GW274150, and montelukast, respectively. There was no difference between AMP reactivity during dosing with GW274150 and placebo (mean doubling dose difference, 0.29; 95% CI, −0.32 to 0.91). There was a trend to inhibition of AMP reactivity during treatment with montelukast compared with placebo (mean doubling dose difference, 0.64; 95% CI, 0 to 1.28).

DISCUSSION

GW274150, a highly selective inhibitor of iNOS, substantially reduced F_{ENO} levels in steroid-naïve patients with asthma. This

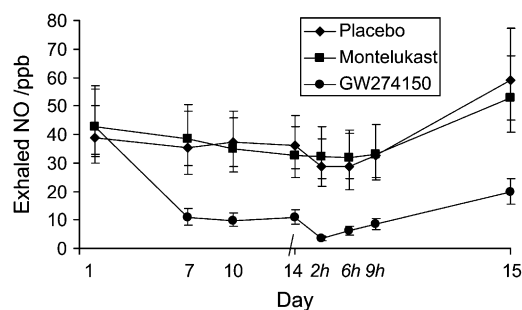


Figure 3. Profile of exhaled NO levels at predose from Days 1 to 14 and then 2, 6, and 9 hours postdose (in italics) on Day 14 after allergen challenge, and then on Day 15.

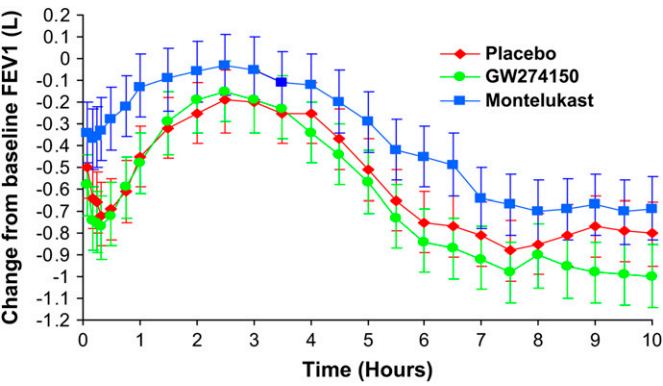


Figure 4. Early and late asthmatic response to inhaled allergen challenge after 14 days of treatment with either GW274150, montelukast, or placebo. Means and 95% confidence intervals of change in FEV₁ compared with post-saline value are shown.

reduction in F_{ENO} levels was not associated with any change in the early or late responses to allergen challenge, or with measurements of bronchial hyperreactivity (AMP reactivity, or methacholine reactivity post-allergen challenge). We have therefore shown that GW274150 was pharmacologically active in the lungs of patients with asthma, but that it was not associated with any beneficial or detrimental effects on a range of airway reactivity measurements over a 14-day dosing period, or on the numbers of inflammatory cells present in BAL.

Selective iNOS inhibitors reduce inflammation in animal models of asthma (19, 27–29). In particular, GW274150 substantially inhibited the allergen-provoked LAR in guinea pigs, whereas in strong contrast the nonselective inhibitor L-NAME exacerbated the response to allergen challenge in these studies (19). Moreover, L-NAME has been shown to exacerbate vascular leakage in response to endotoxin, an effect opposite to that with highly selective iNOS inhibitors in a range of tissues including the lung (30, 31). These observations supported our hypothesis that highly selective iNOS inhibition would be an effective therapeutic strategy in patients with asthma.

GW274150 reduced the levels of F_{ENO} by up to approximately 90%, to levels well below those observed in patients with stable asthma and similar to or lower than those in healthy subjects (32). nNOS and eNOS also contribute to F_{ENO} levels, and the relative contribution of the NOS isoforms is likely to be determined by a variety of factors including genetics, airway inflammation, and environmental stimuli. Nevertheless, our data suggest that iNOS is the major contributing enzyme. The reduction in F_{ENO} in patients with asthma after a single dose of the iNOS inhibitor SC-51 was greater than that observed in the current study, to levels below 2 ppb (25). However, SC-51 is likely to have been inhibiting nNOS at this high dose, because of its modest selectivity for iNOS over nNOS. Caution should be applied in directly comparing

TABLE 4. INDUCIBLE NITRIC OXIDE SYNTHASE AND 3-NITROTYROSINE EXPRESSION IN MUCOSAL BIOPSIES

	Placebo (n = 20)	GW274150 (n = 13)	Montelukast (n = 9)
iNOS intensity	1.2 (0.2)	1.2 (0.1)	1.2 (0.1)
iNOS area, %	9.3 (6.3)	14.8 (9.0)	14.1 (8.3)
3-NT intensity	1.5 (0.2)	1.5 (0.2)	1.6 (0.2)
3-NT area, %	12.1 (11.0)	16.4 (13.7)	21.8 (13.1)

Definition of abbreviations: iNOS = inducible nitric oxide synthase; 3-NT = 3-nitrotyrosine.

Values represent mean (SD).

Intensity of staining in epithelium [(1/optical density) × 100] and stained area as a percentage of epithelial tissue area are shown.

F_{ENO} levels in different studies, as these are dependent on the flow rate and analyzer used, which were different in these two studies (32). It is possible that GW274150 did not completely abolish iNOS activity, although the substantial reduction of F_{ENO} to levels similar to those in healthy subjects (32) suggests that clinically relevant levels of inhibition were achieved. It is also possible that the duration of treatment for 14 days was inadequate to completely inhibit iNOS activity, although the magnitude of the reduction in F_{ENO} levels and the fact that this reached steady state indicate that substantial inhibition of iNOS function was achieved.

Pharmacodynamic steady state inhibition of F_{ENO} by GW274150 was achieved in less than 7 days, which was maintained throughout the EAR (0–2 h) and LAR (4–10 h). Despite effective suppression of NO production, GW274150 did not inhibit allergen reactivity. The inhaled allergen challenge is the most commonly used clinical model for evaluating the effects of potential antiinflammatory therapies for asthma; all the antiinflammatory drugs currently used in clinical practice have beneficial effects in this model (33–36). We considered this model to be an appropriate and sensitive method for assessing the effect of selective iNOS inhibition. We also observed no effect of GW274150 on AMP reactivity, another commonly used and robust method for evaluating antiinflammatory effects, particularly of corticosteroids (37, 38). The lack of any benefit in the methacholine challenge results at 24 hours postallergen reflected the LAR results.

The reaction between NO and superoxide forms peroxynitrite, which has been proposed to mediate a range of biological effects relevant to asthma pathophysiology, such as promoting inflammation and cytotoxicity and increasing bronchial hyperreactivity (9–11). Furthermore, in humans with asthma the levels of airway epithelial iNOS and 3-NT (used as a biomarker for peroxynitrite levels) are increased compared with control subjects (6–8), and correlate with markers of disease severity, such as lung function and bronchial hyperreactivity (6). Inhaled steroids reduce 3-NT and epithelial iNOS expression (6). However, the current study showed that reducing NO levels by selective iNOS inhibition did not reduce 3-NT epithelial expression after allergen

TABLE 3. EARLY AND LATE ASTHMATIC FEV₁ RESPONSES: TREATMENT DIFFERENCES COMPARED WITH PLACEBO

	EAR		LAR	
	Minimal	Weighted Mean	Minimal	Weighted Mean
GW274150	–0.02 (–0.2, 0.16)	–0.02 (–0.16, 0.12)	–0.09 (–0.23, 0.05)	–0.11 (0.23, 0.02)
Montelukast	0.37 (0.19, 0.55)	0.28 (0.15, 0.42)	0.18 (0.04, 0.32)	0.18 (0.06, 0.31)

Definition of abbreviations: EAR = early asthmatic response; LAR = late asthmatic response.

Data represent mean (95% confidence interval) FEV₁ (L).

TABLE 5. INFLAMMATORY CELL COUNTS FROM BRONCHOALVEOLAR LAVAGE POST-ALLERGEN CHALLENGE

	Placebo (n = 23)	GW274150 (n = 16)	Montelukast (n = 9)
Eosinophils, %	4.3 (4.9)	4.7 (4.7)	2.0 (3.3)
Lymphocytes, %	2.1 (4.3)	2.7 (6.7)	2.0 (3.0)
Macrophages, %	89.9 (6.6)	88.0 (9.2)	93.3 (7.5)
Neutrophils, %	3.8 (2.9)	4.6 (4.7)	2.8 (2.8)

Values represent mean (SD).

challenge. This is in direct contrast to a rat model in which GW274150 completely prevented nitrotyrosine staining in the lung tissue after carrageenan induced acute lung inflammation (20). The reason for selective iNOS inhibition having no effect on peroxynitrite bioactivity in asthma may be because a high degree of iNOS inhibition is required under circumstances in which superoxide production is not reduced; in support of this hypothesis, the reaction between NO and superoxide to form peroxynitrite is extremely fast and pH dependent, approaching the diffusion-controlled limit (39). Alternatively it could be because peroxynitrite can be formed by mechanisms other than iNOS (10, 11, 39), or that the allergen challenge itself may be responsible for failing to show a reduction in 3-NT; the allergen challenge may have increased both (1) iNOS activity, and the rise in exhaled NO levels at 24 hours postchallenge supports this theory, and (2) the level of reactive oxygen species, including superoxide, which contribute to 3-NT levels. Finally, it could be that 3-NT in the human lung is long-lived, such that 14 days is insufficient to observe changes. If this is correct, then it argues against the presence of significant denitration enzymes in human lung tissue.

An important group of biologically active nitrogen species produced by iNOS activity are S-nitrosothiols, for example, S-nitrosoglutathione (11–13); the formation of NO can lead to the production of species such as peroxynitrite, which are able to react with free thiols to form S-nitrosothiols. One study in S-nitrosothiol reductase knockout mice has provided evidence that these S-nitrosothiols act as endogenous bronchodilators, decreasing airway reactivity to methacholine (13). In principle, therefore, inhibition of iNOS by GW274150 could decrease the concentration of S-nitrosothiols in the airway and worsen airway hyperreactivity. However, the data in the current study show that airway hyperreactivity is unaffected by iNOS inhibition. Therefore, either S-nitrosothiols are not important bronchodilators in humans, or else alternative sources of these S-nitrosothiols other than iNOS, such as eNOS and nNOS, predominate in the human lung. In addition, the activity of S-nitrosothiol reductase in human airways may be of prime importance in determining S-nitrosothiol levels.

It is known that under some circumstances iNOS can promote prostaglandin E₂ (PGE₂) production through a direct interaction between iNOS and cyclooxygenase 2 (40). Because PGE₂ may be a bronchoprotective mediator in the lungs of patients with asthma, it is possible that any beneficial effects of iNOS inhibition in the airways were counterbalanced by a reduction in PGE₂ levels. We were not able to measure PGE₂ in this study, and therefore were not able to address this possible mechanism to explain the failure of GW274150 to improve the clinical asthma end points studied.

NO has diverse immunological functions in various cell models, ranging from cytotoxicity to immunosuppression (9–12). An immunosuppressive role for NO in asthma could be protective against the development of severe asthma or exacerbations. Coupled with the ability of NO to cause bronchodilation, it is theoretically possible that NO functions mainly as an endogenous protector against pulmonary inflammation and

bronchoconstriction. However, if this were true, then in the current study we would have seen a deterioration in clinical asthma end points after treatment with GW274150. This phenomenon was not observed, suggesting that NO was not functioning as an endogenous protector.

The metabolism of NO in the airways can produce a range of different molecules with diverse effects, including nitrates, nitrites, 3-NT, and S-nitrosoglutathione (9–12). Measuring F_{ENO} alone does not provide a comprehensive assessment of the range of mediators that may be involved in nitrosative stress mechanisms in asthma. We have shown that reducing iNOS activity leads to a reduction in F_{ENO} levels with seemingly no effect on 3-NT expression, but did not address changes in other biologically relevant nitrogen species. However, we did evaluate BAL inflammatory cell counts, and found that a reduction in F_{ENO} levels was not associated with any modulation of inflammatory cell numbers.

Montelukast was used in this study as a “positive control,” which was expected to attenuate the response to the allergen challenge (35, 36). Inhaled corticosteroids could have been used as a positive control (34, 35, 37), but the washout period required would have made a three-part cross-over design less practical. Montelukast did not reduce F_{ENO} levels in our study. The effects of montelukast on F_{ENO} in previous studies have been conflicting, with either a reduction in F_{ENO} observed (41) or no change (42). The variable effect of montelukast on F_{ENO} seems to be less than the effects of inhaled corticosteroids (42).

F_{ENO} monitoring may be used as a measure of asthma control, to guide the need for corticosteroid therapy (43, 44). The current study has shown that the reduction in F_{ENO} levels caused by the selective iNOS inhibitor GW274150 does not lead to any beneficial or detrimental effects on airway hyperreactivity. Although F_{ENO} has applications as a biomarker in asthma, our study provides evidence that selective iNOS inhibition is not an effective therapeutic strategy in asthma.

Conflict of Interest Statement: D.S. received lecture fees, research grants, consultancy fees, and support for conference attendance from various pharmaceutical companies including AstraZeneca, GlaxoSmithKline, Boehringer Ingelheim, and Roche, and received a \$300,000 unrestricted research grant over the last 3 years from GlaxoSmithKline. D.R. is an employee of GlaxoSmithKline. R.G.K. is a full-time employee of GlaxoSmithKline. S.S. is an employee of GlaxoSmithKline. A.W. received consultancy and lecture fees and is the principal investigator on studies for GlaxoSmithKline. A.W. received consultancy fees and was a principal investigator on research studies for Chiesi, and consultancy fees for Novartis and Oriel Therapeutics. S.L. is deceased. B.J.O. had ad hoc consultancy arrangements with several pharmaceutical companies, including GlaxoSmithKline, AstraZeneca, Altana, Aventis, Celgene, Pfizer, and Boehringer Ingelheim, receiving honorariums ranging from \$600 to \$1,200, not exceeding more than \$3,000 in any one year from any company. B.J.O. directs a phase 2 clinical research unit, dedicated to the evaluation of new drugs for airway disease, and his university receives funding appropriate to the complexity of the study at normal commercial market rates. B.J.O. receives speaker's fees for AstraZeneca, GlaxoSmithKline, Pfizer, Boehringer Ingelheim, and Altana, with the honorariums never exceeding \$1,000, and his total annual speaker's fees never exceed \$10,000.

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Selective iNOS inhibition has no effect on allergen challenge in asthma

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ONLINE DATA SUPPLEMENT

Methods

Exclusion Criteria

Exclusion criteria were the presence of other significant medical conditions, known hypersensitivity to montelukast, a respiratory tract infection in the previous 4 weeks, a previous history of a life threatening asthma exacerbation, oral steroid therapy within 5 weeks or inhaled steroid therapy within 1 week of screening, on-going allergen desensitisation and ECG and laboratory abnormalities.

AMP and methacholine challenges

AMP and methacholine challenges were performed using a Mefar Dosimeter (Mefar-Bologna) if the FEV₁ was $\geq 65\%$ predicted. All inhalations were performed slowly from FRC to TLC over 3 seconds followed by breath holding for 6 seconds. There were five inhalations at each dose level. FEV₁ was measured at 60 and 180 secs and the highest recorded. Initially, 0.9% saline control was used. Subjects with a post-saline decrease $\geq 10\%$ on three consecutive occasions did not receive AMP or methacholine.

For AMP, subjects were administered doubling concentrations (DC) from 0.039 to 320mg/ml, until a $\geq 20\%$ fall in FEV₁ was achieved or the highest concentration of AMP was administered. For methacholine, subjects were administered doubling concentrations from 0.03125 to 32 mg/ml until a $\geq 20\%$ fall in FEV₁ was achieved or the highest concentration of methacholine was administered. The provocative concentration required to reduce the FEV₁ by 20% of the post-saline baseline value (PC₂₀) was derived by linear interpolation between the lowest concentration that caused a $>20\%$ fall and the preceding concentration. If the FEV₁ did not fall by more than 20% following the highest concentration then the PC₂₀ was set to the highest concentration

given in the challenge. If the FEV₁ fell by more than 20% following the first concentration the PC20 was derived as [20x lowest concentration]/ [% fall following lowest concentration].

Allergen Challenge

Bronchial allergen challenges were only performed if the FEV₁ was $\geq 65\%$ predicted, and were performed using a Mefar Dosimeter (Mefar-Bologna) as described for AMP and methacholine. Allergen for skin prick tests (Soluprick SQ, Alk Abelló (UK) Ltd) was stored at 4°C; each subject was assessed for sensitivity to house dust mite, cat, grass pollen, and positive and negative controls. The allergen for inhalation was selected according to the largest skin test wheal (positive >3 mm) and clinical history. Fresh solutions of allergen were made up in 0.9% saline in doubling concentrations from 250 SQ-U/ml to 32 000 SQ-U/ml. Subjects inhaled five breaths of saline as control followed by five breaths of the lowest concentration of allergen. FEV₁ was measured at 5 and 10 minutes after inhalation and the highest value recorded for analysis. If the fall in FEV₁ was <10% of the post-saline value, a further five breaths of a quadruple concentration of allergen was inhaled. If a 10–15% fall in FEV₁ was achieved, a further five breaths of a doubling concentration was inhaled. Challenges were terminated when a fall in FEV₁ of at least 15% from the post-saline value was recorded or the maximum concentration had been given. FEV₁ was measured at 15, 20, 30, 45 and 60 minutes and every 30 minutes thereafter for a total of 10 hours after the challenge. The early asthmatic response (EAR) was defined as a fall in FEV₁ of $\geq 20\%$ from the post saline value, on at least one occasion, between 10 and 30 minutes. The late asthmatic response was defined as a fall in FEV₁ of $\geq 15\%$ from the post saline value, on at least three occasions, two of which were consecutive, between 4 and 10 hours after the final concentration of allergen. Minimum LAR was derived as the minimum FEV₁ value over 4-10 hrs post allergen challenge. Minimum EAR was derived as the minimum FEV₁ over 0-2 hrs post allergen challenge. Weighted mean LAR and EAR endpoints were derived by calculating the AUC over the relevant time interval using the linear trapezoidal

rule and dividing by the time interval. The post-saline timepoint (i.e. 0 hrs) was excluded from the derivation of minimum EAR.

FeNO

FeNO was measured using the Ecomedics AG analyser CLD 88 (Ecomedics; Dürnten, Switzerland) calibrated according to the manufacturer's instructions using source gas containing NO at 20 ppm (SIT Analytical, Sandwich, UK) which underwent dilution to a final concentration of 200 ppb. After inhaling NO free air to total lung capacity, subjects exhaled at a constant flow rate against a resistor at a flow of 50 ml/s to determine the plateau NO concentration. Three acceptable readings were recorded from each subject according to ATS criteria and the mean of the three readings was used for analysis.

Bronchoscopy

Subjects were sedated with intravenous midazolam. Topical lignocaine (2-4%) was administered to the upper airways and larynx, followed by passage of a fiberoptic bronchoscope through the nasal passages into the trachea. Up to 6 endobronchial biopsies were obtained from either the right or left lower lobe. Bronchoalveolar lavage was collected from the right middle lobe by instilling a maximum of 4 × 60ml aliquots of pre-warmed sterile 0.9% NaCl solution. The aspirated fluid was stored on ice before filtration (100µm filter, Becton Dickinson). The filtrate was centrifuged (400 g/10 min at 4°C) and the cell pellet washed in RPMI 1640 medium. Viable cell counts were determined by trypan blue exclusion (Neubauer hemocytometer). Cytospin slides were prepared at a concentration of 3×10^4 cells per slide. Slides were stained with Wright's modified Giemsa stain (Sigma Aldrich), and 400 non-epithelial cells per slide were counted.

Immunohistochemistry

Bronchial biopsies were fixed in 10% formaldehyde and, after dehydration, embedded in paraffin wax. Biopsies were cut into 4 µm serial sections for analysis of iNOS and 3-NT. Briefly, following deparaffinisation, the sections were subjected to appropriate antigen retrieval to unmask antigens in formalin fixed and paraffin embedded tissue sections; 0.01M citrate buffer at pH 6.0 in a microwave (10 minutes high and 10 minutes low power) for demonstration of nitrotyrosine, and Antigen Unmasking Solution (Vector Laboratories, Peterborough, UK) in a pressure cooker (for 2 minutes) for the demonstration of iNOS. The sections were treated with 0.3% H₂O₂ in methanol to block endogenous peroxidase, and with Avidin/Biotin Blocking Kit (Vector Laboratories) to block endogenous biotin. Sections were then incubated with a rabbit polyclonal antibody anti- Nitrotyrosine (dilution 1:600; anti-nitrotyrosine rabbit immuno-affinity purified IgG, from Upstate, Chandlers Ford, UK) or with a rabbit polyclonal antibody anti-iNOS (dilution 1:1000; Santa Cruz Biotechnology inc. Heidelberg, Germany). The nitrotyrosine antibody used was the rabbit polyclonal antibody which has been used to demonstrate and localise tyrosine nitration in human lung in several papers [E1-6]. Viera et al [E6] have described in detail a series of controls that have been carried out to establish the specificity of this antibody: antibody binding was blocked by co-incubation with nitrotyrosine or nitrated bovine serum albumin but not by amino-tyrosine, phospho-tyrosine, or bovine serum albumin. Reduction of tissue nitrotyrosine to amino-tyrosine by sodium hydrosulfite also blocked antibody binding. In control specimens with no overt pulmonary disease, there was only slight or no staining.

The secondary antibody biotinylated anti rabbit IgG was then applied, followed by Vectastain Elite Avidin Biotin Complex with Horseradish Peroxidase as the label (both Vector Laboratories) and demonstrated using Diaminobenzidine (Dako, Ely, UK) as the chromogen. Sections were counterstained with Carazzi's haematoxylin, dehydrated, cleared and mounted in Pertex.

The slides were scanned using the NanoZoomer fast scanner (Hamamatsu Photonics, Welwyn Garden City Herts, UK) using the 20x objective, providing microscopy images with a resolution of 0.23µm per pixel. The images were then subjected to image analysis using PharmaView software (Medical Solutions plc, Nottingham, UK). For tissues immunostained for iNOS and 3-Nitrotyrosine, the epithelial layer was manually identified. Separate thresholds were set for pixels representing positive (brown) immunostaining, and for pixels representing areas unstained tissue.

Statistical Analysis

The primary objective was to evaluate the effect of treatment with GW274150 or montelukast compared to placebo on the LAR. The secondary objectives were to perform the same comparisons for active treatments against placebo for methacholine reactivity following allergen challenge, AMP challenge, the EAR, FeNO and biomarkers of iNOS activity from bronchoscopy. The sample size calculation was mainly based on the primary endpoint; The minimum LAR change compared to the post saline FEV₁ measurement. Previous allergen challenge data showing that the minimum LAR change during placebo treatment had a mean of -1.108L and a within-subject standard deviation of 0.532 allowed the following sample size estimation;. In order to detect a 50% attenuation (i.e. improvement) in the minimum LAR compared to placebo with 90% power, at the 5% significance level, a sample size of 22 subjects were required. This sample size was also sufficient to detect a 2 doubling dose difference between active and placebo treatments for the AMP and methacholine challenges. 28 patients were recruited so that at least 22 would complete the study.

The post saline value was fitted as a covariate fixed effect. It was decided a priori that methacholine and AMP PC₂₀ data and baseline would be log₂-transformed prior to analysis in order to interpret the results as a doubling dose difference. FeNO change from baseline ratio was analysed using repeated measures analyses. One analysis was carried out on the day 7, 10 and

14 pre-dose data, a separate analysis was carried out including the day 14 time-points. Mixed effects models were fitted with period, treatment group, baseline (pre-dose day 1), planned relative time, treatment group by planned relative time interaction and baseline by planned relative time interaction terms fitted as fixed effects, and subject fitted as a random effect. FeNO change from baseline ratio and baseline data was log_e-transformed prior to analysis due to the distribution of the data. All other data were summarised only. The analyses were carried out using SAS version 8.2 on a UNIX platform.

Results

Table E1 Summary of FeNO Levels (ppb)

Day	Time	Placebo (N= 28)		GW274150 90mg (N=26)		Singulair (N=25)	
		n	Geom. Mean (95% CI)	n	Geom. Mean (95% CI)	n	Geom. Mean (95% CI)
1	Pre-dose	28	38.8 (30.0, 50.1)	25	42.9 (32.9, 55.9)	25	42.8 (32.0, 57.1)
7	Pre-dose	26	35.3 (25.9, 48.0)	26	10.8 (8.3, 14.0)	25	38.4 (29.1, 50.6)
10	Pre-dose	26	37.2 (28.6, 48.3)	26	9.8 (7.8, 12.3)	24	35.0 (26.7, 45.8)
14	Pre-dose	26	36.1 (27.9, 46.8)	25	11.0 (8.7, 13.8)	25	32.7 (25.0, 42.7)
	2h post-dose	25	28.9 (21.8, 38.3)	24	3.4 (2.8, 4.2)	25	32.4 (24.5, 42.7)
	6h post-dose	25	28.9 (20.7, 40.3)	25	6.2 (4.8, 7.9)	25	32.0 (24.6, 41.7)
	9h post-dose	25	32.5 (24.2, 43.5)	24	8.4 (6.7, 10.4)	25	32.8 (24.7, 43.7)
	24h post-dose	25	59.1 (45.2, 77.2)	25	19.6 (15.7, 24.6)	25	52.6 (40.9, 67.7)

Immunohistochemistry

Examples of 3-NT and iNOS staining are shown in Figure E1. A range of staining intensity in different subjects are shown.

Fig E1A

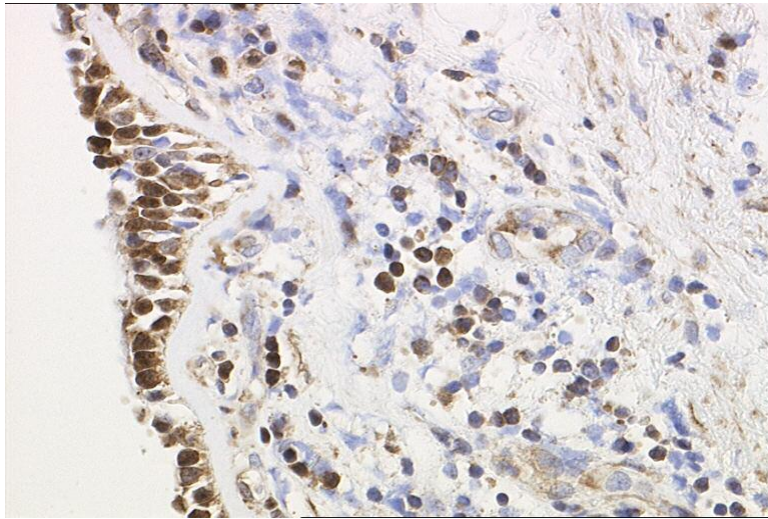


Fig E1B

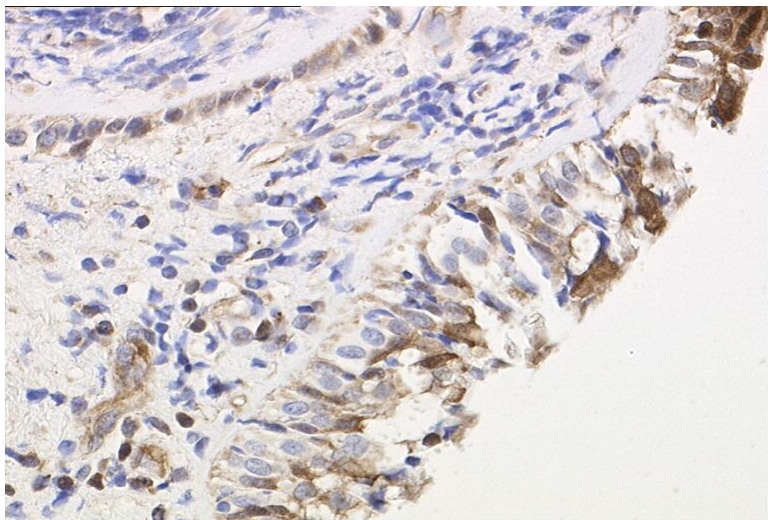


Fig E1c

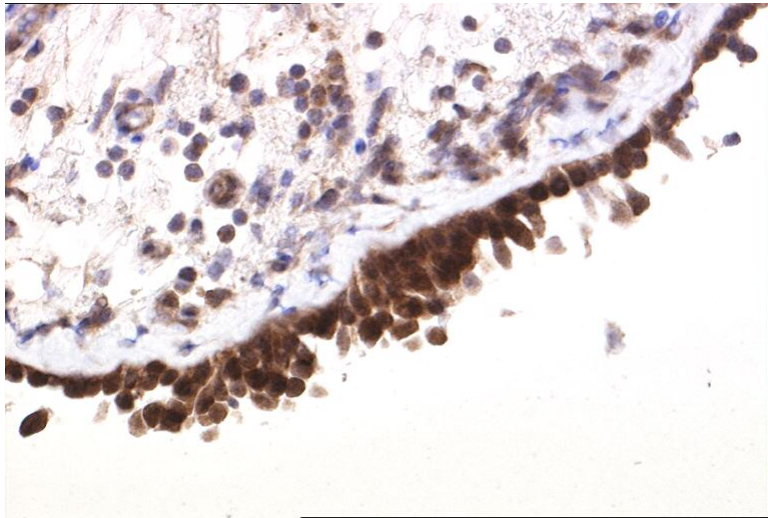
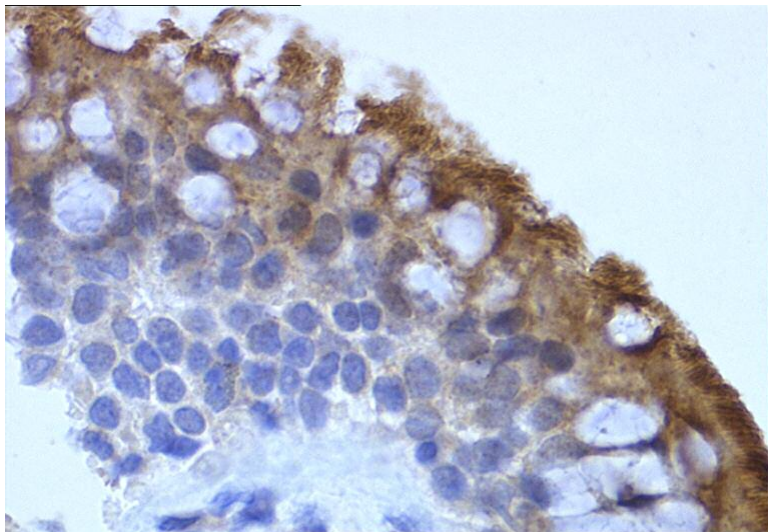


Fig E1d



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Figure Legends

Fig E1. Representative images for the immunohistochemical analysis of iNOS and 3-NT expression within 4µm thick sections of human lung tissue. 3,3'-diaminobenzidine (DAB) detection (brown) of the rabbit polyclonal antibody anti-iNOS showing (a) strong epithelial iNOS staining and (b) weak epithelial iNOS staining. DAB detection of the rabbit polyclonal antibody anti-nitrotyrosine showing (c) strong 3-NT staining (d) weak 3 NT staining.