Leukotriene C₄ synthase polymorphisms and responsiveness to leukotriene antagonists in asthma

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Aim Cysteinyl leukotrienes are important pro-inflammatory mediators in the pathogenesis of asthma, while leukotriene C₄ synthase is a key enzyme in their biosynthesis. Our aim is to evaluate whether responsiveness to leukotriene receptor antagonists was determined by expression of the variant (C) or wild-type (A) polymorphism of this enzyme.

Methods We carried out a retrospective analysis of 8 randomised, placebo-controlled trials performed in our department in mild-to-moderate asthmatics. In all trials, effect of leukotriene receptor antagonist was compared to placebo, where the primary outcome was bronchial hyperresponsiveness to adenosine monophosphate or methacholine. Secondary outcomes were forced expiratory volume in 1 second, exhaled nitric oxide and peripheral blood eosinophils.

Results For the primary outcome of attenuation of bronchial hyperresponsiveness by leukotriene receptor antagonist *vs* placebo, there were significant effects within each genotype on adenosine monophosphate (AMP) ($n = 78$): 2.21 and 2.07-fold improvements for AA and AC/CC, respectively; while for methacholine ($n = 81$) there were 1.39 and 1.36-fold improvements, respectively. There were no significant differences between genotypes (i.e. AA *vs* AC/CC): geometric mean fold-differences of 1.07 (95%CI 0.63–1.81) and 1.02 (95%CI 0.70–1.50) for AMP and methacholine, respectively. There were also no differences between genotypes for all secondary outcomes.

Conclusion Polymorphisms of leukotriene C₄ synthase did not determine responsiveness, in terms of attenuation of bronchial hyperresponsiveness, to leukotriene receptor antagonists in mild-to-moderate asthmatics. Further prospective large pharmacogenetic studies are required in more severe patients, where there may be greater improvements in pharmacodynamic outcome measures such as bronchial hyperresponsiveness and exhaled nitric oxide.

Keywords: asthma, bronchial hyperresponsiveness, leukotriene receptor antagonist

Introduction

Cysteinyl leukotrienes are important pro-inflammatory mediators implicated in the pathogenesis of asthma, resulting in bronchoconstriction, inflammatory cell recruitment and smooth muscle proliferation [1]. Blockade of this pathway with leukotriene receptor antagonists (LTRAs), demonstrates improved clinical outcome mea-

sures when given as first or second-line therapy [2]. Similar to most asthma therapies, patients tend to exhibit variability in clinical response to LTRAs of which individual pharmacogenetics may play an important role.

Leukotriene C₄ (LTC₄) synthase, which is expressed in airway inflammatory cells, is an integral enzyme in the biosynthesis of cysteinyl leukotrienes converting leukotriene A₄ to C₄. A bi-allelic polymorphism of the LTC₄ synthase gene has been discovered within the putative promoter region, characterised by an adenosine (A) to cytosine (C) transversion at a position 444 bp upstream of the initiation codon (A-444C). In aspirin intolerant asthmatics, overactive transcription of variant C polymorphism is associated with enhanced expression of LTC₄

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synthase in peripheral blood eosinophils [3]. Furthermore, enhanced synthesis of leukotriene C₄ by calcium ionophore stimulated blood eosinophils has been demonstrated in genotypes containing variant (C) compared with wild-type (A) polymorphisms [4]. In the same study, it was demonstrated in 23 asthmatics that treatment with zafirlukast (a LTRA) 20 mg twice daily for 2 weeks resulted in a numerically greater response in forced expiratory volume in one second (FEV₁) comparing genotypes AC or CC *vs* AA.

Conventional end-points such as lung function provide little information regarding the underlying asthmatic disease process, and tend to be downstream markers of airway calibre rather than inflammation. Bronchial hyper-responsiveness (BHR) is a useful surrogate marker of airway inflammation and can be performed using direct bronchoconstrictor stimuli such as methacholine and indirect stimuli such as adenosine monophosphate (AMP), with the latter considered to have more clinical relevance to asthmatic inflammation [5, 6].

The aim of our study was to evaluate whether clinical response to LTRAs was determined by asthmatics expressing genotypes containing variant (C) or wild type (A) polymorphisms of LTC₄ synthase. To achieve this, we carried out a retrospective analysis of randomised controlled clinical trials in which LTRA was compared to placebo in terms of attenuating BHR and effects on inflammatory surrogates.

Methods

Study design and patients

This retrospective genotype analysis was of 8 randomised, placebo-controlled, crossover, chronic dosing trials in mild-to-moderate persistent asthmatics per-

formed in our department (Table 1) [7–14]. References 9 and 10 were sponsored by MSD (who make montelukast) (Merck Inc., Whitehouse Station, New Jersey, USA) and reference 12 was sponsored by Aventis (who make triamcinolone) (Aventis Inc., Bridgewater, New Jersey, USA); all other studies received no funding from the pharmaceutical industry. All patients gave written consent to participation and genotyping, which was approved by the Tayside committee on medical research ethics. In each study, the primary endpoint was BHR to AMP or methacholine for LTRA *vs* placebo. We also examined treatment effects on exhaled nitric oxide (NO), peripheral blood eosinophils and FEV₁.

Measurements and assays

The methacholine and AMP bronchial challenge tests were performed using standardised methods, as previously described [15, 16]. The methacholine provocative dose to cause a 20% fall in FEV₁ (PD₂₀) or provocative concentration of AMP to cause a 20% fall in FEV₁ (PC₂₀) was determined by computer-assisted interpolation of the log dose–response curve. Exhaled NO was measured using a LR2000 NO gas analyser (Logan Research, Rochester, UK) as described elsewhere [17]. Spirometry was performed according to the American Thoracic Society [18] criteria using a Vitalograph Compact spirometer (Vitalograph Ltd, Bucks, UK) which was calibrated daily. Eosinophil count was measured using a SE-9000 Haematology analyser (Sysmex UK Ltd, Bucks, UK). The A/C single nucleotide polymorphism at –444 of the LTC₄ synthase promoter was determined by the presence (C) or absence (A) of a MspI restriction site after digestion and agarose gel electrophoresis of a 563-bp polymerase chain reaction product of genomic DNA as previously described [19].

Table 1 Study demographic details.

Reference	Mean FEV ₁	Design	Duration	ICS	Primary outcome	NO	Eosinophils	FEV ₁	Number in study
Wilson [7]	79%	ML <i>vs</i> SM <i>vs</i> PL	2 weeks	√	AMP: ML > PL,	NS	S	NS	20
Lipworth [8]	76%	ZAF <i>vs</i> FM <i>vs</i> PL	1 weeks	√	methacholine: ZAF > PL	S	ND	NS	24
Sims [9]	79%	ML <i>vs</i> FM <i>vs</i> PL	2 weeks	√	AMP: ML > PL	NS	S	NS	15
Currie [10]	81%	SM/ML <i>vs</i> SM/PL	2 weeks	×	methacholine: ML > PL	NS	NS	S	20
Wilson [11]	91%	ML <i>vs</i> BUD <i>vs</i> PL	2 weeks	×	AMP: ML > PL	S	NS	NS	12
Dempsey [12]	96%	ML <i>vs</i> TA <i>vs</i> PL	4 weeks	×	methacholine: ML > PL, AMP: ML > PL	NS	NS	NS	21
Dempsey [13]	92%	ICS/ZAF <i>vs</i> ICS <i>vs</i> ICS/THE <i>vs</i> PL	4 weeks	√	methacholine: ZAF > PL	S	ND	S	24
Wilson [14]	81%	CZ/ML <i>vs</i> BUD <i>vs</i> PL	2 weeks	×	AMP: ML > PL	NS	NS	S	14

Abbreviations: AMP, adenosine monophosphate; CZ, cetirizine; FM, formoterol; ICS, inhaled corticosteroid; ML, montelukast; ND, not done; NO, nitric oxide; NS, nonsignificant; PL, placebo; S, significant; SM, salmeterol; TA, triamcinolone; THE, theophylline; ZAF, zafirlukast.

Data analysis

The AMP PC₂₀, methacholine PD₂₀, NO and eosinophil data were logarithmically transformed to normalise their distributions prior to analysis. Data were subsequently analysed using a statgraphics statistical software package (STSC Software Publishing Group, Rockville, MD, USA). An overall analysis of variance was performed followed by multiple-range testing with Bonferroni correction, set at 95% confidence limits (two-tailed, $P < 0.05$) for both within and between genotype effects. Due to the small numbers who were homozygous for the variant allele (CC) who had an AMP or methacholine challenge, we grouped together the genotypes having a variant allele (CC and AC) for comparison with AA, as has been performed previously [4, 20].

Results

Demographic details of the individual studies are shown in Table 1. The allelic frequencies for LTC₄ synthase polymorphisms were 71% for wild-type A and 29% for variant C. The genotype frequencies were AA in 49%, CC in 6% and AC in 44%. The within-genotype responses showed significant ($P < 0.05$) effects of LTRA *vs* placebo for the primary outcome variable of BHR to AMP or methacholine, as indicated by 95% CI which excluded unity. Heterogeneity across the trials was not significant ($P = 0.08$; Chi squared value = 14.2).

Values for all studies irrespective of inhaled corticosteroids, for LTRA *vs* placebo response from run-in values (Table 2), the response for AMP and methacholine was

significant ($P < 0.05$) within genotype for both AA and AC/CC. For NO the within genotype response was significant ($P < 0.05$) for AA alone, while for eosinophils, the response was significant for both genotypes.

For effects irrespective of concomitant treatment with inhaled corticosteroids, the differences between AA *vs* AC/CC in terms of BHR, NO and eosinophils were not significantly different (Table 2). FEV₁ (as change from placebo) showed a significant ($P < 0.05$) within-genotype response for AC/CC ($n = 71$): 0.15 L (95% CI 0.30–0.27) but not AA ($n = 69$) 0.11 L (–0.01–0.24). There was no significant difference between genotypes AA *vs* AC/CC: 0.04 L (–0.21–0.13).

For studies where LTRA were given without concomitant inhaled corticosteroids, the geometric mean fold differences (95% CI) for difference between AA *vs* AC/CC for AMP, methacholine, NO and eosinophils were 1.01-fold (0.46–2.19), 1.28-fold (0.64–2.56), 0.94-fold (0.75–1.17) and 0.92-fold (0.78–1.09), respectively. For studies with concomitant inhaled corticosteroids, the corresponding differences were 1.16-fold (0.56–2.2), 0.93-fold (0.59–1.48), 0.92-fold (0.70–1.22) and 0.93-fold (0.60–1.27), respectively. There were no differences in the distribution of responders compared to nonresponders for each genotype for either AMP or methacholine (figure and Table 3).

Discussion

We have demonstrated that polymorphisms of LTC₄ synthase did not determine treatment response to LTRAs in terms of surrogate inflammatory markers including

	Geometric mean fold difference for LTRA <i>vs</i> placebo (95% CI)		Geometric mean fold difference between genotypes (95% CI)
	AA	AC/CC	
AMP	2.21 (1.56–3.13), $n = 44$	2.07 (1.39–3.08), $n = 34$	1.07 (0.63–1.81)
Methacholine	1.39 (1.04–1.85), $n = 35$	1.36 (1.06–1.75), $n = 46$	1.02 (0.70–1.50)
Nitric oxide	0.87 (0.76–0.99), $n = 69$	0.94 (0.83–1.07), $n = 71$	0.92 (0.77–1.11)
Eosinophils	0.81 (0.72–0.90), $n = 46$	0.89 (0.80–0.99), $n = 47$	0.90 (0.78–1.05)

For responses within each genotype group, the 95% CI which excludes unity denote a significant effect for a given variable. Abbreviations: AMP, adenosine monophosphate; LTRA, leukotriene receptor antagonist.

Table 2 Within and between genotype effects (irrespective of concomitant inhaled corticosteroid therapy).

	AMP		Methacholine	
	AA	AC/CC	AA	AC/CC
Number (%) of patients with > 1 doubling dose improvement	23 (52%)	15 (44%)	14 (40%)	16 (35%)
Number (%) of patients with < 1 doubling dose improvement	21 (48%)	19 (56%)	21 (60%)	30 (65%)

Table 3 Responders *vs* nonresponders for each challenge according to genotype.

Figure 1 Individual doubling dilution shift with mean values in AMP PC₂₀ between leukotriene receptor antagonist *vs* placebo in patients with wild type (A) or variant (C) polymorphisms. The doubling dose dilution shift is calculated as $\log_{10}$ difference in PC₂₀ for LTRA minus placebo divided by $\log_{10}2$ (i.e. 1 doubling dilution shift = a 2 fold shift). Corresponding data as means and 95% CI for the geometric mean fold shift are given in Table 2.

BHR. We feel our choice of primary endpoint is particularly relevant as BHR is a relatively upstream marker of the asthmatic inflammatory process. This is in contrast to conventional tests of airway calibre such as FEV₁, which are measures of airway function rather than inflammation. It may not be surprising to discover that a single allelic variation does not determine response to LTRAs as cysteinyl leukotrienes are synthesized via a cascade of enzymes. This in turn implies that a variety of polymorphisms may be responsible for the variation in effects of LTRA between different patients. Our data showed significant improvements for within genotype effects, which were larger for AMP than methacholine challenge. This is probably due to cysteinyl leukotrienes being released from primed mast cells following AMP challenge whereas methacholine acts directly upon airway smooth muscle [6]. It is relevant to mention that there were differences in duration of treatment between different studies. For example, in reference 8 the effect of zafirlukast *vs* placebo was significant after only 1 week on the primary outcome variable of BHR to methacholine, which we therefore felt justified its inclusion. In this study, the mean shift was 0.6 of a doubling dose. This compares to a 0.5 doubling dose shift in reference 12 after 2 weeks,

and a 0.6 doubling dose shift in same study after 4 weeks. In other words, there was little difference between 1 and 4 weeks on BHR to methacholine. Indeed, even single doses of montelukast have been shown to attenuate BHR [21].

Asano *et al.* evaluated the effects of pranlukast for 4 weeks in 48 patients with moderate-to-severe persistent asthma [20]. Patients with AC/CC had a significantly greater bronchodilator response in FEV₁ compared to patients with AA. Indeed, inspection of individual data from Asano *et al.* broken down according to responders (> 10% improvement in FEV₁) *vs* nonresponders to pranlukast, revealed that the responders had a significantly lower baseline FEV₁ (55% *vs* 70%, $p < 0.01$). However, this merely may have reflected baseline airway calibre, as the percentage FEV₁ reversibility to albuterol was also greater in pranlukast responders (27% *vs* 14%, $p < 0.01$). The calculated odds ratio in the study by Asano was 8.1 (95% CI: 1.1–58.7) for responsiveness to pranlukast when comparing genotypes, although the CI indicates that the variability was large. In our patients who had asthma of mild-to-moderate severity, the magnitude of FEV₁ response was small and so it is not possible to draw any meaningful conclusions on bronchodilator responsiveness. Furthermore, our studies were all powered on BHR as the primary outcome and the patients had only mild impairment of lung function. Studies in more severe asthmatics (i.e. FEV₁ < 60% predicted) would be useful to assess bronchodilator effects, as greater improvement of airway calibre would be possible. However, these would preclude performing bronchial challenge tests for safety reasons.

Irrespective of genotype, LTRAs do give an overall beneficial effect in terms of bronchoprotection. A meta-analysis of 13 trials evaluated the overall effect on LTRAs on BHR, which demonstrated that chronic dosing results in an overall estimated protection of almost 1 doubling dose/dilution [22]. Furthermore, when BHR is assessed using AMP compared to methacholine, van den Berge demonstrated that improvements in AMP PC₂₀ were more closely correlated with markers of airway inflammation, while methacholine was related to airway calibre [23].

It is important to point out that inhaled corticosteroids have little effect upon the biosynthesis of cysteinyl leukotrienes [24, 25]. In these two studies, there was no effect of prednisolone and fluticasone propionate, respectively, upon leukotriene E₄ levels after allergen challenge. This, therefore, explains why there are additive effects of LTRAs to inhaled corticosteroids. In another study [26] in corticosteroid treated asthmatics, there was an additive effect on BHR to AMP plus blood eosinophils and exhaled NO. In other words, there is a nonsteroidal mediated component to BHR which can be attenuated

by leukotriene blockade, however, it is reasonable to presume that one would expect the signal to be greater in steroid naive patients.

In conclusion, our data suggests that LTC₄ synthase polymorphisms do not determine the in responsiveness to LTRAs in mild-to-moderate asthmatics. Further prospective large pharmacogenetic studies are required in more severe patients, where there may be greater improvements in pharmacodynamic outcome measures such as bronchial hyperresponsiveness and exhaled NO. It is however, conceivable that LTC₄ synthase may determine the bronchodilator response to LTRAs but not anti-inflammatory response, as cysteinyl leukotrienes are potent bronchoconstrictors. Similarly, studies in more severe asthmatics would be required to further evaluate this hypothesis.

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