

Effects of cysteinyl leukotrienes in small human bronchus and antagonist activity of montelukast and its metabolites

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Summary

Background Evidence suggests that small airways contribute to clinically significant processes in asthma. Cysteinyl leukotrienes (CysLTs) are considered to be pivotal mediators in the pathogenesis of asthma. Montelukast (MK), a specific CysLT₁ receptor antagonist, is metabolized in two main hydroxylated metabolites (termed M5 and M6, respectively).

Objectives The aims of this study were to compare the responsiveness of small and large human bronchi to the three CysLTs, to evaluate the antagonist activity of MK, M5 and M6 in these preparations of human bronchi, and to characterize the CysLT receptors involved in the contractile response.

Methods and results In isolated small bronchus (i.d. 0.5–2 mm), the potencies (–log molar EC₅₀) of LTC₄, LTD₄ and LTE₄ were 9.3 (*n* = 11), 9.1 (*n* = 30) and 8.4 (*n* = 14), respectively. The three CysLTs were about 30-fold more potent in small bronchi than in larger bronchi (i.d. 4–6 mm). In small bronchi, MK significantly shifted to the right the CysLT concentration–effect curves with pA₂ values against LTC₄, LTD₄ and LTE₄ of 9.1 (*n* = 3), 9.0 (*n* = 11) and 8.7 (*n* = 5), respectively. The antagonist potencies of M6 and M5 were similar to MK and fivefold lower, respectively. A similar activity of MK against the three CysLTs suggested that CysLT₁ receptors are involved in the contraction of human bronchus. Analysis by RT-PCR also indicated that human bronchus mainly expressed CysLT₁ receptors.

Conclusion MK exerts a potent antagonist activity against the particularly potent constricting effects of CysLTs in isolated human small bronchi, which only expressed the CysLT₁ receptor subtype. The metabolites of MK are also potent *in vitro* antagonists, but may not participate in the therapeutic activity of MK due to their low plasma concentrations in patients treated with the recommended dose of MK.

Keywords cysteinyl leukotrienes, cysteinyl leukotriene receptors, montelukast, small human bronchus
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Introduction

Cysteinyl leukotrienes (CysLTs) (leukotriene C₄ (LTC₄), LTD₄ and LTE₄) are potent pro-inflammatory lipid mediators that are produced by many inflammatory cells: eosinophils, macrophages, basophils and mast cells. These CysLTs cause smooth muscle constriction and proliferation, secretion of mucus, promote eosinophil recruitment and activation, and increase microvascular permeability [1, 2]. For these reasons, CysLTs are considered to be pivotal inflammatory mediators that contribute to the pathogenesis of asthma. CysLTs are potent and long-lasting bronchospastic agents in human airways both *in vivo* and *in vitro* and possess considerably greater potency than histamine or methacholine [3–7]. It has been predicted that much of the resistance to airflow seen in asthma or during agonist challenges is due to

contraction of small (< 2 mm i.d.) airways [8, 9]. Very few *in vitro* studies have compared these functionally significant small airways with larger airways, even though significant pharmacological and mechanical differences have been reported between airways from different parts of the bronchial tree in animals [10, 11] as well as in humans [12–15]. In these latter studies, the contractile responses of small bronchi were found to be either stronger for neurokinin A [12], and endothelin-1 [14] or weaker for isoprostanes [15] than the corresponding responses of larger bronchi. The direct comparison of the potency and efficacy of the CysLTs has not been carried out in small and in large human bronchi.

There is pharmacological evidence for the presence of at least two CysLT receptors (CysLT₁ and CysLT₂) in human lung [16, 17]. Recently, the possible existence of a third type of CysLT receptor (CysLT₃) has been suggested in the human pulmonary artery [18]. The CysLT₁ and CysLT₂ receptors have recently been cloned [19–22]. Pharmacological evidence suggests that CysLT₁ receptors mediate the airway smooth muscle constriction as well as the inflammatory effects

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induced by the CysLTs [1, 2]. Accordingly, many potent and selective CysLT₁-receptor antagonists have been developed [23, 24] and three have been marketed for asthma treatment (montelukast (MK), pranlukast and zafirlukast). After oral administration, MK causes prolonged, potent antagonism of LTD₄-induced bronchoconstriction in patients with asthma [25]. The *in vitro* antagonist activity of MK has been evaluated in binding studies on cloned human CysLT₁ receptors, on guinea-pig and sheep LTD₄ receptors in lung membranes, and in functional studies on isolated guinea-pig trachea [21, 22, 26]. MK is extensively metabolized into six derivatives. Only the 21- and 36-hydroxylated metabolites (named M5 and M6, respectively) were detected in the plasma after oral administration of MK to healthy volunteers. M6 binds to LTD₄ receptors on guinea-pig lung membranes nearly as avidly as MK, while M5 binds about 10-fold less (MSD pre-clinical data). The leukotriene receptor population in guinea-pig lung has been shown to be heterogeneous and different from its human counterpart [27, 28]. However, no functional studies have been performed on human isolated airways to evaluate the antagonist activity of MK as well as of the two hydroxylated metabolites M5 and M6.

The main objectives of the present study, therefore, were to compare the agonist potency and efficacy of the CysLTs on large and small human bronchi, to evaluate the antagonist activity of MK and its two active metabolites on these preparations, particularly on the functionally important small bronchi, and to characterize the CysLT receptors expressed on this human tissue.

Methods

Isolated preparations

Samples of human lung were obtained from thirty males (age range 38–83 years) and six females (age range 49–81 years) who had undergone lobectomy or pneumectomy for lung carcinoma. Small bronchi (internal diameter: 0.5–2 mm) and large bronchi (internal diameter: 3–6 mm) were carefully removed from the macroscopically normal regions of the diseased lung and dissected free of surrounding parenchyma and blood vessels. The bronchi were placed either in cooled (4°C) Krebs–Henseleit solution for organ bath studies or quickly frozen in liquid nitrogen and stored at –80°C for testing with RT-PCR.

Human isolated bronchi contractions

Bronchi were set up in 10-mL organ baths containing Krebs–Henseleit solution (composition in mM: NaCl 118, KCl 5.4, CaCl₂ 2.5, MgSO₄ 0.6, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 11.7, pH 7.4) maintained at 37°C and continuously gassed with a mixture of 95% O₂ and 5% CO₂. Bronchi were set up as ring preparations under different initial loads (small diameter bronchi, 1.5 g; large diameter bronchi, 2.5 g) to ensure an optimal response to the contractile agonists. Isometric force transducers (UF-1, Pioden, Canterbury, UK) and two-channel recorders (Linseis E200, Polyabo, Strasbourg, France) were used to monitor the changes in force. The preparations were washed with fresh buffer every

15 min over a 60-min equilibration period. At the beginning of each experiment, a maximally effective concentration of acetylcholine (3 mM) was administered to identify the maximal contractile response. After washing and recovery of the basal tone, the bronchi were again equilibrated for 60 min before addition of leukotrienes to the organ bath. Concentration–response curves for each LT (LTC₄, LTD₄ and LTE₄) were constructed by cumulative increases in the bath concentration of CysLTs, with each concentration remaining in contact with the tissue until the response reached a plateau before addition of the subsequent concentration. Only one cumulative concentration–response curve was generated with each bronchial ring. Where appropriate, antagonists or vehicle were administered to the tissue baths 45 min before the addition of CysLTs. In a series of experiments, rings from paired small and large bronchi were incubated in the presence of a 5-lipoxygenase inhibitor (AA861, 10 µM) or the vehicle for 45 min before cumulative addition of LTD₄. No inhibitor of the metabolism of CysLTs (γ-glutamyltranspeptidase inhibitors or aminopeptidase inhibitors) was added to the Krebs–Henseleit solution since previous studies have shown that, in contrast with the results in guinea-pig isolated trachea, these inhibitors did not improve the contractile response of human bronchi to LTC₄ or LTD₄ [4, 7, 16, 17, 27].

RT-PCR

These reactions were performed as previously described [29]. Total RNA of approximately 30 mg of human bronchi was isolated according to the method of Chomczynski and Sacchi [30]. Residual genomic DNA was removed by incubating the RNA samples with RNase-free, fast protein liquid chromatography (FPLC) pure DNase I (Amersham Biosciences, Essex, UK) and RNasin (Promega Corp., Madison, WI, USA). First-strand cDNA was synthesized using Moloney murine leukaemia virus reverse transcriptase and random hexamers according to the manufacturer's instructions (First-strand cDNA synthesis kit, Amersham Biosciences). The resulting cDNA samples were amplified by PCR using a DNA thermal cycler (MJ Research, Watertown, MA, USA) and specific oligonucleotide primers designed with the analysis software Primer 3 (Rozen and Skaletsky, 2000, code available at <http://www.genome.wi.mit.edu/genomesoftware/other/primer3>). The primer pairs used were: (a) CysLT₁R, forward 5'-AGCCCCACAAGACAATCAA-3' and reverse 5'-AGGAGAGGGTCAAAGCAACAA-3', based on the published sequence of the human CysLT₁ receptor [21], and designed to amplify a PCR product of 358 base pairs (bp), and (b) CysLT₂R, forward 5'-GCAACCATCCATCTCCGTATC-3' and reverse 5'-CCAGGAAACGCACAACACTC-3', based on the human CysLT₂R cDNA sequence [20], giving a PCR product of 392 bp. Amplification of the human β-actin gene transcript was used to control the efficiency of RT-PCR among the samples. Sequences of forward and reverse primers for β-actin were 5'-TCCCTGGAGAAGAGCTACGA-3' and 5'-ATCTGCTGGAAGGTGGACAG-3', respectively, based on the published sequence of the human gene [31]. The expected size of the PCR product was 362 bp. PCR mixes contained 0.2 µM primers, 1.5 U of *Taq* polymerase (Amersham Biosciences), the buffer supplied, 2.5 mM MgCl₂, 200 µM dNTPs and cDNA in 25 µL. Cycle numbers were 32

for CysLT₁R, 35 for CysLT₂R and 25 for β -actin. After a hot start (2 min at 94 °C), the profile for each cycle was 94 °C for 10 s; 60 °C for 20 s and 72 °C for 30 s. Serial half dilutions of cDNA were amplified at the indicated number of cycles for each leukotriene receptor and β -actin to ensure analysis of products in the linear range of amplification. The PCR products were separated by gel electrophoresis on 1.7% agarose, stained with ethidium bromide and visualized and photographed under a UV transilluminator (Spectronics Corp., New York, NY, USA). Band intensities were scanned by densitometry using a video documentation system and the image analysis software Intelligent Quantifier (BioImage Systems Corp., Ann Arbor, MI, USA). mRNA levels for the three tachykinin receptors and β -actin were analysed on each tissue, with each RT-PCR assay being performed in triplicate. Amplicon sizes were verified by comparison with a DNA mass ladder and the identity of each PCR product was established by DNA sequence analysis.

Expression of the results and statistical analysis

The changes in force produced by the CysLTs were expressed as percent of the response to acetylcholine (3 mM). The maximal contractions in response to acetylcholine were 4.4 ± 0.1 g in small bronchi ($n = 141$) and 4.3 ± 0.2 g in large bronchi ($n = 72$). All numerical data are expressed as arithmetic means $\pm$ standard error of the mean (SEM). pD_2 values were determined for each cumulative concentration–response curve as the negative logarithm of the molar EC_{50} value (the concentration of agonist inducing a contraction which was 50% of the maximal contraction). The apparent dissociation constant (pK_B value) was calculated using the following equation [32]: $K_B = [B]/DR - 1$, where $[B]$ is the concentration of antagonist (MK-476, M5 or M6) and DR (dose ratio) is the ratio of EC_{50} of agonist (LTC₄, LTD₄ or LTE₄) in the presence and absence of antagonist. pK_B values represent the negative logarithm of K_B . pA_2 values of antagonists were calculated according to Arunlakshana and Schild [33]. The competitive nature of the antagonism was evaluated by the Schild plot method [33] on data obtained with at least three concentrations of antagonist between 10^{-9} and 10^{-7} M tested against LTC₄, LTD₄ or LTE₄.

Differences between concentration–response curves were tested using ANOVA for repeated measures followed by Bonferroni–Dunn's t -test if required. Probability values (P) for ANOVA for repeated measures correspond to concentration–treatment interactions. Differences between means were determined using Student's t -test; $P < 0.05$ were considered significant.

Drugs and solutions for functional studies

The CysLTs (LTC₄, LTD₄ and LTE₄) were purchased from Cayman (SPI-Bio, Massy, France) in methanol, stored at -80°C , and diluted with Krebs–Henseleit solution. Montelukast (MK-476; [R-(E)]-1-[[[1-[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl]thio]methyl]cyclopropane acetic acid, monosodium salt) and its two metabolites M5 (21-hydroxy analog) and M6 (36-hydroxy analog) were a generous gift of Dr Ford-Hutchinson (Merck Frosst Canada Inc., Dorval, Quebec,

Canada). Zafirlukast was kindly provided by Sanofi-Synthelabo Recherche (Montpellier, France). Zafirlukast was dissolved with DMSO/water (0.1/0.9 ml) for the 10^{-5} M concentration and then diluted with distilled water. The maximal final concentration of DMSO was 0.1% and this concentration did not alter the maximal response to acetylcholine or LTD₄. Acetylcholine and AA861 (2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone) were from Sigma (St Quentin Fallavier, France). MK, M5 and M6 as well as acetylcholine were dissolved in distilled water. AA861 was dissolved in ethanol (10^{-2} M solution) and then diluted with distilled water.

Results

CysLT-induced contractions

Concentration–effect curves for the different CysLTs are presented in Fig. 1 and Table 1. These results were derived from experiments in which no prior drug treatments were performed. In small or large bronchi preparations, the potencies (pD_2 values) were not significantly different among these agonists. However, CysLTs were about 30-fold more potent in small bronchi (potency 1.5-log higher). The maximal effects of CysLTs were significantly higher in small vs. large bronchi except for LTE₄, which induced a similar

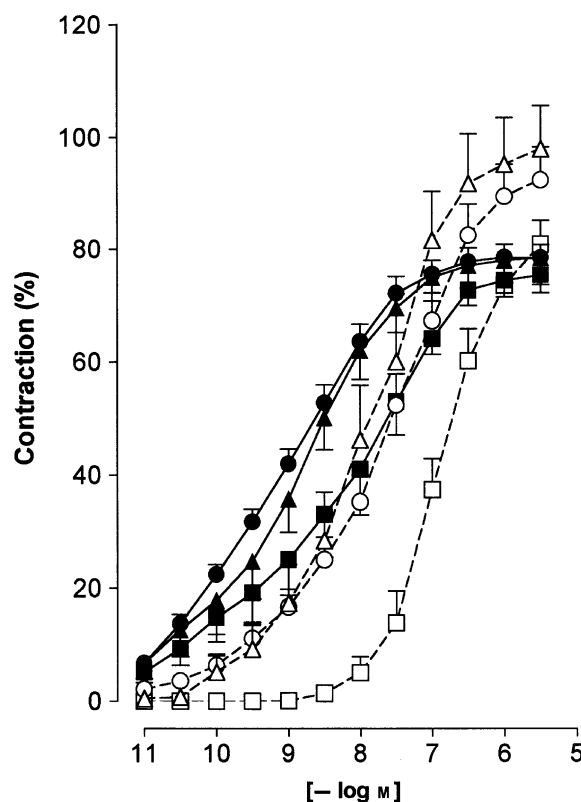


Fig. 1. Log concentration–response effects for LTC₄ (▲, △), LTD₄ (●, ○) and LTE₄ (■, □) in producing contractions of human small airways (filled symbols) and large airways (empty symbols, dotted lines). Results are expressed as percentages of maximum contractions induced by 3 mM acetylcholine. Vertical lines represent SEM, which are not shown when they lie within the limits of the symbol or overlap one another. A summary of these experiments can be found in Table 1.

maximal effect in large bronchi (Fig. 1). Pre-treatment with AA861 (10 μ M) induced a 0.3 log-rightward shift of the LTD₄ concentration–effect curves in both small and large bronchi preparations ($n = 5$ paired preparations), with no alteration of the difference in LTD₄ potency between small and large bronchi (pD_2 in small bronchi: 8.9 ± 0.3 and 8.5 ± 0.3 ; pD_2 in large bronchi: 7.8 ± 0.2 and 7.3 ± 0.3 , in the absence or presence of AA861, respectively).

Antagonism of CysLT-induced contraction of human bronchi

MK and its two metabolites (M5 and M6) caused parallel rightward shifts of CysLT concentration–response curves. The effects (pK_B) of MK, M5 and M6 (10^{-7} and 10^{-8} M)

against LTC₄-, LTD₄- and LTE₄-induced contractions of human small bronchus are summarized in Fig. 2 and Table 2. For the three antagonists, the pA_2 values (Table 2) against each CysLT have been calculated for a series of experiments performed on paired (adjacent) small bronchus segments incubated with at least three different concentrations of antagonists ranging from 10^{-9} to 10^{-7} M. Schild plot analysis of the data produced slopes that were not statistically different from 1 (MK: 0.90 ± 0.09 , 0.90 ± 0.06 and 1.14 ± 0.07 ; M5: 1.03 ± 0.15 , 1.04 ± 0.17 and 1.19 ± 0.06 ; M6: 1.02 ± 0.11 , 1.00 ± 0.05 and 1.00 ± 0.07 ; for LTC₄, LTD₄ and LTE₄,

Table 2. Effect of montelukast and its two hydroxylated metabolites (M5 and M6) on the contractions induced by the three cysteinyl leukotrienes in human small isolated bronchi preparations

Drugs	LTC ₄ response		LTD ₄ response		LTE ₄ response	
	<i>n</i>	pK_B or pA_2	<i>n</i>	pK_B or pA_2	<i>n</i>	pK_B or pA_2
Montelukast						
10^{-8} M	3	9.0 ± 0.4	11	9.0 ± 0.2	5	8.8 ± 0.2
10^{-7} M	6	8.9 ± 0.2	13	8.9 ± 0.2	7	8.6 ± 0.2
	3	$9.1 \pm 0.2^*$	11	$9.0 \pm 0.1^*$	4	$8.7 \pm 0.1^*$
M6						
10^{-8} M	5	9.0 ± 0.2	6	9.4 ± 0.2	4	9.1 ± 0.2
10^{-7} M	7	8.9 ± 0.2	5	8.9 ± 0.3	5	8.9 ± 0.3
	5	$9.2 \pm 0.3^*$	6	$9.4 \pm 0.2^*$	4	$8.9 \pm 0.2^*$
M5						
10^{-8} M	4	8.5 ± 0.3	5	8.7 ± 0.2	3	7.9 ± 0.5
10^{-7} M	6	8.4 ± 0.1	5	8.6 ± 0.2	4	8.0 ± 0.3
	4	$8.5 \pm 0.3^*$	5	$8.8 \pm 0.3^*$	3	$8.1 \pm 0.3^*$

Table 1. Cysteinyl leukotriene-induced contractions in small and large human isolated bronchi preparations. Responses are expressed as pD_2 ($-\log$ molar EC₅₀) and $E_{\max}$ (force expressed in percentages of maximum contraction induced by 3 mM acetylcholine). Values are reported as mean $\pm$ SEM for the number of preparations presented in parentheses

Agonist	pD_2 values		$E_{\max}$ (%)	
	Small bronchus	Large bronchus	Small bronchus	Large bronchus
LTC ₄	$9.3 \pm 0.3^{\dagger}$ ($n = 11$)	7.9 ± 0.2 ($n = 5$)	$75.5 \pm 4.0^*$ ($n = 11$)	98.0 ± 7.6 ($n = 5$)
LTD ₄	$9.1 \pm 0.1^{\dagger}$ ($n = 30$)	7.9 ± 0.2 ($n = 20$)	$78.4 \pm 2.3^*$ ($n = 30$)	92.4 ± 5.9 ($n = 20$)
LTE ₄	$8.4 \pm 0.3^{\dagger}$ ($n = 14$)	7.0 ± 0.1 ($n = 9$)	75.4 ± 3.1 ($n = 14$)	80.9 ± 4.0 ($n = 9$)

* $\dagger$ Denotes a value that is statistically different from that obtained in large bronchi (*Student's *t*-test or Mann–Whitney *U*-test; $\dagger$ ANOVA for repeated measures).

Antagonist activities are expressed either as pK_B ($-\log$ molar K_B) for 10^{-8} and 10^{-7} M of each compound or pA_2 . Values are reported as mean $\pm$ SEM for the number of preparations presented in parentheses.

* pA_2 values.

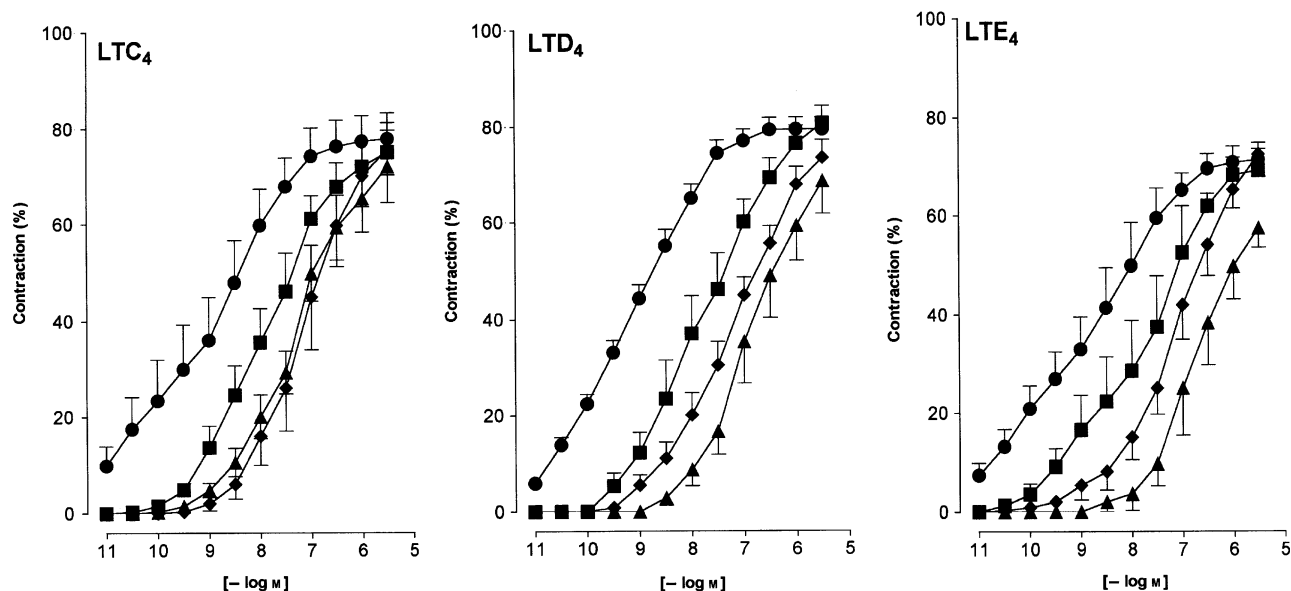


Fig. 2. Log concentration–response effects for LTC₄, LTD₄ and LTE₄ in producing contractions of human small airways in the absence (●) and presence of MK (◆), M5 (■) or M6 (▲) at 10^{-7} M. Results are expressed as percentages of maximum contractions induced by 3 mM acetylcholine. Vertical lines represent SEM, which are not shown when they lie within the limits of the symbol or overlap one another. A summary of these experiments and other similar experiments can be found in Table 2.

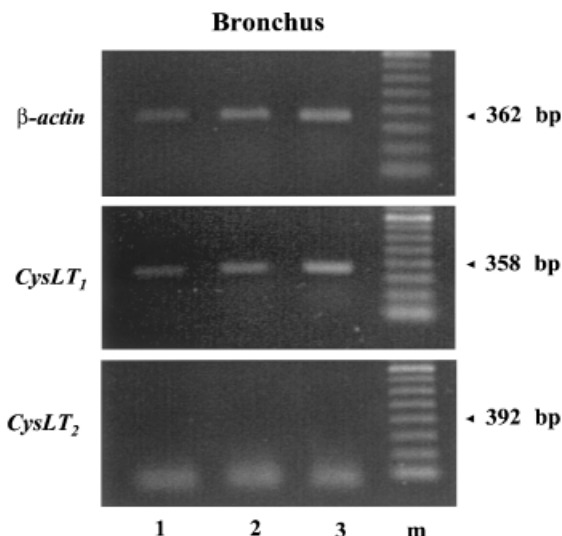


Fig. 3. Agarose gel showing products of RT-PCR assay for cDNA from human bronchi. After normalization to β -actin mRNA levels, serial half dilutions of cDNA were amplified for 25 (β -actin), 32 (CysLT₁R) or 35 (CysLT₂R) cycles with specific oligonucleotide primers. Lane 1 represents the more diluted sample in each series. A single transcript corresponding to the size predicted for the CysLT₁ receptor was detected but the specific band corresponding to the CysLT₂ receptor was only detected after amplification of higher amounts of bronchial cDNA. m, molecular size standards. Data are representative of typical results in six different bronchi.

respectively), indicating competitive antagonism for the three compounds against each leukotriene. The pK_B and pA_2 values for M5 were about fivefold lower than for MK and M6, suggesting a weaker antagonist activity. Under the same experimental conditions, the pA_2 value for zafirlukast against LTD₄ was 10.0 ± 0.3 ($n = 8$) in small bronchi. In the large bronchi, the pK_B values of montelukast (10^{-7} M) against LTC₄-, LTD₄- and LTE₄-induced contractions were 8.5 ± 0.3 ($n = 3$), 8.7 ± 0.3 ($n = 7$) and 8.9 ± 0.5 ($n = 3$), respectively.

RT-PCR studies on human bronchus

A typical example of RT-PCR products from human bronchi ($n = 6$) is illustrated in Fig. 3. The sequence of the CysLT₁ receptor PCR fragment was identical to that previously published (nucleotide positions 524–881 bp). The fragment amplified for the CysLT₂ receptor had the expected size (392 bp) and its sequence was identical to that previously published (nucleotide positions 24–415 bp). We only detected the 358 bp product encoding the CysLT₁ receptor. However, amplification of a high amount of cDNA, corresponding to 1 μ g of total RNA, permitted visualizing a low abundance transcript for the CysLT₂ receptor (not shown), suggesting that this receptor is present only in a trace amount in the human bronchi. In all experiments, the two negative controls yielded no detectable products, indicating that (i) all reagents were free of target sequence contamination and (ii) the RT-PCR products do not come from contaminating genomic DNA.

Discussion

The first finding of the present study is that CysLTs are about 30-fold more potent on isolated small human bronchi than on

large bronchi. Our results on large bronchi are similar to the results previously reported on this human preparation [4, 17, 27]. In comparison with these latter studies, a recent investigation on the mechanism of LTD₄-induced constriction of human bronchioles (300–500 μ m internal diameter) suggested that the potency of LTD₄ ($pD_2 = 9.2$) may be at least 10-fold higher in these small airways than in larger human airways [7]. The present study definitely demonstrates the much higher potency (about 30-fold) of the three CysLTs on human small airways than on large airways. Under resting conditions, the tone of human isolated bronchi is largely the result of contractile 5-lipoxygenase products [34] and may influence the *in vitro* contractile response to CysLTs. However, 5-lipoxygenase inhibition with AA861 [35], while reducing LTD₄ potency in both small and large bronchi, did not influence the magnitude of the potency difference between small and large airways. Therefore, the higher potency of CysLTs in small bronchi is not related to differences in LT-dependent inherent tone between small and large airways. Epithelium removal has been previously shown to induce a weak leftward shift of the LTD₄ concentration–response curve without alteration of the maximum response and without alteration of the responses to LTC₄ or LTE₄ [28]. In addition, the bronchial epithelium does not appear to be the source of CysLTs. [34]. It is therefore unlikely that differences in epithelial function may explain the difference in potency between small and large bronchi. Comparisons of the potency of different constricting agents in large and small human isolated bronchi have shown various results. The potencies of endothelin-1 and neurokinin A have been found to be 10-fold higher in small airways [12, 14, 36], whereas the potency of histamine was similar in small and large airways [13] and the potencies of 8-iso-PGE₁ and 8-iso-PGE₂ were even four fold to 25-fold higher in large airways [15]. The reasons for these differences in potency have not been investigated, but differences in the distribution of ionic channels and currents in smooth muscles from human small bronchioles and main bronchus may explain these variations [37].

With respect to the potencies of these constricting agents, the CysLTs appear to be the most potent agonists in human small airways. This result is of interest since physiological and pathological evidence, from techniques such as high-resolution computed tomography, histologic analysis on resected lung tissue, autopsy lung specimen and transbronchial biopsies, has reinforced the concept that inflammation in asthma is not merely confined to the large central airways but also extends to the entire bronchial tree (for reviews, see [9, 38, 39]). Several studies indicated that inflammatory changes in the small airways are similar but more severe than in large airways of asthmatic patients [40–42]. For example, a greater number of activated eosinophils (EG2 positive) are present within the small airways (<2 mm internal diameter) than within the large airways [42] and, in patients with nocturnal asthma, only alveolar and distal airway (and not central airway) eosinophilia correlated with overnight reduction in lung function [43]. It should be noted that eosinophils produce CysLTs and that, in asthmatic patients with nocturnal exacerbations, urinary LTE₄ at night was significantly higher than in patients without nocturnal attacks or in control subjects [44, 45]. It has also been proposed that distal airway inflammation contributes to other clinically significant

processes in asthma. In asymptomatic asthmatic patients with normal spirometric values, peripheral airways resistance has been shown to be increased by more than sevenfold when compared to control individuals, and these measurements correlated with responsiveness to methacholine and to exercise [46]. In addition, patients with mild asthma who had frequent exacerbations had increased closing volumes and closing capacities, compared with patients with similar pulmonary function who did not experience frequent exacerbations, indicating that distal airways inflammation may be the cause of difficult-to-control asthma [47]. It is noteworthy that CysLT production is increased during exercise-induced asthma and asthma exacerbations [48–50]. Since the CysLTs exert more potent constricting effects on small airways than on large airways, all these results reinforced the role of CysLTs in nocturnal asthma, in exercise-induced asthma as well as in asthma exacerbations.

Although the distal airways are significant and persistent sites of inflammation in asthma, inflammation in this lung compartment appears to be undertreated with most current inhaled corticosteroids [39]. CysLTs antagonists are administered by the oral route and can therefore inhibit the contractile and pro-inflammatory effects of these mediators in both central and peripheral airways. Several studies have shown that this class of anti-asthma drug was efficient in preventing exercise-induced asthma and in improving asthma control with a reduction in the number of exacerbations and awakenings due to asthma [1, 24]. MK is a potent and specific antagonist on cloned human CysLT₁ receptor [21, 22] without significant activity on cloned human CysLT₂ receptor [19, 20]. MK has been shown to inhibit LTD₄ binding to guinea-pig lung and to antagonize contractions of guinea-pig trachea induced by LTD₄ with a pA₂ of 9.3, whereas it was completely inactive vs. contraction induced by LTC₄ [26]. In both large and small human bronchi, MK antagonized the contractions induced by the three CysLTs with a similar activity. This result is in good agreement with studies conducted with other CysLT₁-receptor antagonists [16, 27, 28], and supports conclusions that human bronchus contains a uniform population of CysLT₁ receptors involved in airway smooth muscle contraction. Furthermore, the antagonist potency of MK in human small bronchus was similar to the potency against LTD₄-induced contraction in guinea-pig trachea [26] and against LTD₄-induced calcium mobilization or activation of pigment dispersion in human CysLT₁-receptor transfected cells [19, 21, 22]. Under our experimental conditions, zafirlukast (ICI-204,219) was found to be slightly more potent than MK against LTD₄-induced contraction in small airways. However, in binding studies, the activity of MK is not affected by the presence of human serum albumin, whereas the activity of zafirlukast is, to some extent, inhibited in the presence of plasma proteins [26]. Therefore, in addition to differences in bioavailability and in pharmacokinetics, the absence of plasma protein in our experimental system precludes any translation of this *in vitro* result to a potential higher clinical efficacy of zafirlukast [25].

MK has been evaluated for its activity in attenuating inhaled LTD₄-induced bronchoconstriction in patients with asthma [25]. In this latter study, the median fold shifts vs. placebo in the concentration of LTD₄ required to produce a

50% fall in specific airway conductance (PC₅₀ sGaw) were in the range of 56–85 for doses of MK associated with plasma concentrations of about 100 ng/mL, that is 10^{−7} M [25]. For the same concentration of MK, the median shift of the LTD₄ concentration–response curve on isolated human small bronchi was 71 (present study, data not shown). The similar level of antagonist activity in human isolated bronchus and in LTD₄ bronchial challenge suggests that MK may have a high lung bioavailability and that the *in vitro* results may translate into clinical efficacy. The peak plasma concentration of MK observed 3 h following oral administration of 10 mg was in the range of 400–500 ng/mL corresponding to about 7 × 10^{−7} M [51, 52]. Such a level of plasma concentration was associated with a more than 100-fold median shift vs. placebo of LTD₄ PC₅₀ sGaw [25]. Pharmacodynamic studies with β-blockers and H₁-receptor antagonists suggest that a 20-fold shift of the dose–response curve results in clinical benefit [53, 54]. After repeated oral administration of 10 mg MK, the plasma concentrations at trough were nearly constant ranging from 18 to 24 ng/mL, corresponding to about 3 × 10^{−8} M [52]. This level of concentration caused a 29-fold rightward shift of LTD₄ concentration–response curves on human isolated bronchus (*n* = 4, data not shown), suggesting that, 20–24 h after oral administration of the recommended dose of montelukast, the level of protection afforded by this dose may be beyond the clinically significant threshold inhibition of LTD₄-induced bronchoconstriction. The two hydroxylated metabolites (M5 and M6) detected in plasma following oral administration of 100 mg MK in healthy human subjects were found to be either as potent as the parent drug for M6 or about fivefold less potent for M5 against CysLT-induced contraction of human isolated bronchus. In adult and paediatric subjects treated with oral therapeutic doses of MK, the plasma levels of these metabolites were below the detection limit (20 ng/mL, i.e. 3 × 10^{−8} M) and have been estimated at least 10-fold lower than the plasma concentrations of MK (MSD preclinical data). It is therefore unlikely that these two active metabolites participate in the clinical activity of MK.

The presence of mRNA transcripts specific for the CysLT₁ receptor was detected in all bronchi, whereas amplification of a high amount of cDNA was required to visualize a low abundance transcript for the CysLT₂ receptor, demonstrating the relative low level of expression of the CysLT₂ subtype in human bronchus. The low level of CysLT₂ receptor expression in lung explains the absence of detection of CysLT₂ receptor mRNA in human lung and trachea in a previous study [55]. This result confirms the functional analysis conducted with MK (present study) or other CysLT₁ antagonists [27, 28], and indicates definitely that the CysLT receptor involved in the contraction of human bronchus is mainly of the CysLT₁ subtype. In addition, analysis of the *in situ* expression of CysLT₂ receptor mRNA in human lung has shown that the main expression of this receptor was seen in interstitial macrophages with distinctly weaker expression in smooth muscle cells [20]. In human pulmonary vein, smooth muscle CysLT₂ receptors are involved in the contractile response to CysLTs [17]. However, since the contraction of human bronchus is mainly mediated by CysLT₁ receptors (present study, [27, 28]), CysLT₂ receptors expressed in human airway smooth muscle may represent functional

receptors not linked to bronchoconstriction but involved in smooth muscle proliferation [56].

In conclusion, the results of the present *in vitro* investigation indicate that (1) CysLTs are 30-fold more potent in small human airways than in large airways, (2) MK is a potent antagonist of the three CysLTs in both the small and large human bronchi, with pA_2 in the nanomolar range, (3) the two hydroxylated metabolites of MK are also potent LT antagonists, but may not participate in the therapeutic activity of MK due to their low plasma concentrations in patients treated with the recommended dose of MK, and (4) human bronchus mainly expresses the CysLT₁ receptor subtype.

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