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Pharmacological characterization of the cysteinyl-leukotriene antagonists CGP 45715A (iralukast) and CGP 57698 in human airways *in vitro*

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1 Cysteinyl-leukotrienes (cysteinyl-LTs) are important mediators in the pathogenesis of asthma. They cause bronchoconstriction, mucus hypersecretion, increase in microvascular permeability, plasma extravasation and eosinophil recruitment.

2 We investigated the pharmacological profile of the cysteinyl-LT antagonists CGP 45715A (iralukast), a structural analogue of LTD_4 and CGP 57698, a quinoline type antagonist, in human airways *in vitro*, by performing binding studies on human lung parenchyma membranes and functional studies on human isolated bronchial strips.

3 Competition curves vs [^{3}H]-LTD_4 on human lung parenchyma membranes demonstrated that: (a) both antagonists were able to compete for the two sites labelled by [^{3}H]-LTD_4; (b) as in all the G-protein coupled receptors, iralukast and CGP 57698 did not discriminate between the high and the low affinity states of the *CysLT* receptor labelled by LTD_4 ($K_{i1} = K_{i2} = 16.6$ nM $\pm$ 36% CV and $K_{i1} = K_{i2} = 5.7$ nM $\pm$ 19% CV, respectively); (c) iralukast, but not CGP 57698, displayed a slow binding kinetic, because preincubation (15 min) increased its antagonist potency.

4 In functional studies: (a) iralukast and CGP 57698 antagonized LTD_4-induced contraction of human bronchi, with pA_2 values of 7.77 $\pm$ 4.3% CV and 8.51 $\pm$ 1.6% CV, respectively, and slopes not significantly different from unity; (b) the maximal LTD_4 response in the presence of CGP 57698 was actually increased, thus clearly deviating from apparent simple competition.

5 Both antagonists significantly inhibited antigen-induced contraction of human isolated bronchial strips in a concentration-dependent manner, lowering the upper plateau of the anti-IgE curves.

6 In conclusion, the results of the present *in vitro* investigation indicate that iralukast and CGP 57698 are potent antagonists of LTD_4 in human airways, with affinities in the nanomolar range, similar to those obtained for ICI 204,219 and ONO 1078, two of the most clinically advanced *CysLT* receptor antagonists. Thus, these compounds might be useful drugs for the therapy of asthma and other allergic diseases.

Keywords: Cys-leukotriene; LTD_4; human lung parenchyma; human bronchi; receptor; antagonist; binding; CGP 45715A; iralukast; CGP 57698

Introduction

The Cys-leukotrienes (cysteinyl-LTs) C_4, D_4 and E_4 are the products of the oxidative metabolism of arachidonic acid through the activity of 5-lipoxygenase (Murphy *et al*., 1979; Samuelsson, 1983). A variety of inflammatory cells synthesize cysteinyl-LTs in response to biological and non-biological stimuli (Drazen & Austen, 1987): eosinophils, basophils and mast cells (Lewis & Robin, 1985; Thien & Walters, 1995) are able to synthesize cysteinyl-LTs from arachidonic acid, but cysteinyl-LTs can also be produced through transcellular metabolism from neutrophil-derived LTA_4 by vascular endothelial cells (Feinmark, 1990; Feinmark & Cannon, 1986; Maclouf *et al*., 1989) and platelets (Maclouf & Murphy, 1988).

LTD_4 acts via G-protein mediated recepters (Mong *et al*., 1987; Crooke *et al*., 1989) generating bronchoconstriction, mucus hypersecretion and pro-inflammatory effects such as increase in microvascular permeability. Moreover LTD_4 induces plasma exudation and consequent oedema by opening gaps between endothelial cells (Dahlén *et al*., 1981), and promotes eosinophil migration (Foster & Chan, 1991). Furthermore, their role in modulating the activity of nerve function and smooth muscle proliferation in the airways has been recently evaluated (see Hay *et al*., 1995 for review). These effects may contribute to airway hyper-responsiveness, mucus plug formation, epithelial cell damage and other changes in airway morphology. For these reasons, cysteinyl-LTs are at present considered to be pivotal inflammatory mediators that contribute to the pathogenesis of asthma.

These findings have stimulated many pharmaceutical companies to develop programmes aimed at the discovery of cysteinyl-LT antagonists, either based on the structure of LTD_4 or unrelated to it, to provide therapeutic agents for use in asthma.

Iralukast and CGP 57698 are two recently developed potent cysteinyl-LT antagonists that have been shown to be active in various animal models, both *in vitro* and *in vivo* (Bray *et al*., 1990; von Sprecher *et al*., 1991a,b; 1996; 1997).

In the present paper we describe the *in vitro* pharmacological profile of iralukast and CGP 57698 in human airways, as

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determined by binding studies on human lung parenchyma membranes (HLPM) and functional studies on human isolated bronchial strips.

Methods

Reverse-phase high performance liquid chromatography of cysteinyl-LTs

Reverse-phase h.p.l.c. of cysteinyl-LTs was routinely carried on to determine ligand purity before use in *in vitro* studies. Only leukotrienes with a purity grade higher or equal to 90% were used. Samples were injected onto a C18 Ultrasphere-ODS column and eluted isocratically with a mixture of methanol/water/acetic acid (65:35:0.02, pH 5.8) as previously described (Rovati *et al*., 1992).

Preparation of human lung membranes

Crude membranes were prepared from macroscopically normal specimens removed at thoracotomy for lung cancer as previously described (Rovati *et al*., 1985; 1992). Briefly, specimens were minced and homogenized in 10 mM HEPES buffer, pH 7.4 (1:24, w:v), centrifuged for 10 min at $770 \times g$ and 4°C and the supernatant centrifuged for 20 min at $27{,}000 \times g$ and 4°C. The pellet was resuspended, centrifuged again and finally resuspended in 1/20 of the homogenization volume. Aliquots were prepared and stored at −80°C until use, for not more than three months. The preparation was performed at 4°C in 10 mM HEPES buffer, pH 7.4.

[^{3}H]-LTD$_4$ binding in human lung parenchyma membranes

Equilibrium binding experiments were performed in polypropylene tubes (LP Italiana, S.p.A., Milan Italy) at 25°C for 30 min. Under these conditions, [^{3}H]-LTD$_4$ binding attained equilibrium even at the lowest concentrations used. HLPM were preincubated with the receptor antagonist for 15 min before the addition of [^{3}H]-LTD$_4$, unless otherwise specified. HLPM (0.25 mg protein/sample), 1 mM $CaCl_2$, 10 mM cysteine, 10 mM glycine and 10 mM HEPES buffer pH 7.4 and LTD$_4$ at the indicated concentrations were added to the incubation mixture to achieve a final volume of 250 μl. Bound ligand was separated from unbound ligand by rapid vacuum filtration (Brandel cell harvester) onto 2.5% polyvinylalcohol soaked fibre glass filter paper (Whatman GF/C), followed by washing with 2×4 ml of HEPES buffer at 4°C. Radioactivity was measured by liquid scintillation counting (Filter Count, Packard). Non-specific binding was calculated by LIGAND (see Data analysis below) as one of the unknown parameters of the model and ranged between 40 and 50% of the total binding of 0.5 nM [^{3}H]-LTD$_4$.

Design of binding experiments

Displacement, mixed and multiligand binding curves were designed and performed to study the interaction of iralukast, CGP 57698, ICI 204,219 and ONO 1078 with the *CysLT* receptor. Homologous LTD$_4$ curves were always performed according to a mixed type protocol, combining both saturation (the first 5 concentrations in the curves, 0.01 – 0.5 nM) and displacement (the last 3 concentrations, 10 – 1000 nM) curves (Rovati *et al*., 1991). By effectively combining both saturation and competition protocols in a single curve, high concentrations of the ligand can be reached without the need for excessive amounts of labelled ligand (competition part of the curve), yet have adequate radioactivity in the lower concentration range (saturation part of the curve). To study the interaction of the heterologous ligands with the *CysLT* receptor, 0.5 nM [^{3}H]-LTD$_4$ was used in displacement studies. However, this approach allowed us only to evaluate the interaction of the unlabelled ligand with the lower affinity class of the receptors (Rovati, 1993). To simultaneously evaluate the interaction of the antagonists with the two affinity states of the *CysLT* receptor, we performed a series of LTD$_4$ mixed curves, in the absence or presence of the indicated concentration of the antagonist within the same experiment, in accordance with the multiligand protocol (Rovati *et al*., 1990). A multiligand design potentially includes all possible combination of concentrations of labelled and unlabelled ligand and thus may be regarded as a family of dose-response curves for a ligand in the presence of increasing concentrations of a second ligand. This type of protocol is necessary because, to study an unlabelled ligand (regardless of its K_i) with a classical heterologous competition curve, one must use a concentration of the labelled ligand lower than its K_d, otherwise one incurs in the so call 'self-displacement' by the same labelled ligand, thus preventing the unlabelled ligand from interacting with that site (Rovati, 1993). On the other hand, such a low concentration of labelled ligand might yield an amount of bound radioactivity extremely low. To overcome this limit, we extensively used the multiligand protocols. Binding is expressed as the ratio of bound concentration over total ligand concentration (B/T) vs the logarithm of total concentration. Total concentration is the sum of 'hot' and 'cold' ligand and includes non-specific binding.

Human isolated bronchi preparation

Macroscopically normal human bronchi (inner diameter: 2 to 3 mm) were obtained from patients undergoing thoracic surgery for pulmonary carcinoma and prepared as described by Bolla *et al*. (1997). Briefly, the specimens were placed in cold (4°C) saline solution and studied within 120 min from resection. The bronchi were carefully dissected free of surrounding parenchyma and blood vessels, helically cut and prepared as strips 2 to 3 mm wide and about 10 mm long. The bronchial strips were suspended in 5 ml organ baths containing Tyrode solution (composition in mM: NaCl 140, KCl 3, $CaCl_2$ 1, $MgCl_2$ 0.05, NaH_2PO_4 0.5, glucose 8.4 and $NaHCO_3$ 12) (Dahlén *et al*., 1980) maintained at 37°C, bubbled with 95% O_2 – 5% CO_2, pH 7.4. Contractions were measured with a Basile 7004 isometric force transducer and recorded on a Basile Gemini 7070 polygraph. Bronchial strips were set at an initial tension of 1 g weight and washed with fresh buffer every 15 min over a 60 min equilibration period. At the beginning of the experiment, cumulative concentrations (10 – 300 μM) of acetylcholine (ACh) were administered to check the sensitivity of the preparations and to identify their maximal contractile response. After washing and recovery of the basal tone, the bronchi were equilibrated with either the antagonists or their vehicles and challenged with either LTD$_4$ or antigen (see subsequent paragraphs).

Antagonism of LTD$_4$-induced contraction of human isolated bronchial strips

Bronchial strips were treated with 3 mM L-cysteine (Sok *et al*., 1980) to inhibit LTD$_4$ metabolism. After 15 min, the indicated concentration of antagonist (10 nM – 1 μM) or the vehicle

dimethyl sulphoxide (DMSO) was added. Fifteen minutes later, cumulative concentration-response curves were obtained by increasing the concentration of LTD_4 (0.1 nM – 3 μM). Only one LTD_4 concentration-response curve was obtained from each bronchial strip. The contractile response to each concentration of LTD_4 was expressed as % of the maximal response to 300 μM ACh.

Antagonism of antigen-induced contraction of human isolated bronchial strips

To make the contractile response specifically cysteinyl-LT dependent, isolated tissues were incubated in the presence of an antihistamine (mepyramine, 0.3 μM) and indomethacin (10 μM). After ACh-induced contraction and recovery of the basal tone, strips were treated for 15 min with the indicated concentration of the antagonist and then challenged with cumulative concentrations (0.01 – 3.0 μg ml^{-1}) of a polyclonal antibody specific for human IgE ε-chain (Anti-IgE) (Viganò *et al*., 1990). Only one anti-IgE concentration-response curve was obtained from each strip.

Contractions were expressed as % of the maximal response to 300 μM ACh.

Data analysis and statistical evaluation

Analysis of equilibrium ligand binding data was performed by means of a computer program, LIGAND (Munson & Rodbard, 1980). A series of models of increasing complexity, e.g., one or two binding sites, was considered. Statistical analysis of the concentration-response curves was performed by using the computer program ALLFIT (De Lean *et al*., 1978), which calculates the lower and upper plateaux, the slope and the EC_{50} and allows the comparison of two or more curves. Selection of the best fitting model and evaluation of the statistical significance of the parameter difference was based on the F test for the extra sum of square principle (Draper & Smith, 1966). A statistical level of significance of $P<0.05$ was accepted. Antagonist potency was evaluated according to Arunlakshana and Schild (1959). All the curves shown were generated by computer. (LIGAND, ALLFIT).

Drugs and chemicals

[^{3}H]-LTD_4 (127 – 173 Ci mmol^{-1}) was purchased from Dupont NEN (Boston, MA, U.S.A.). LTD_4, CGP 45715A (iralukast; ((1**R**,2**S**)-1-hydroxy-1-(3-trifluoromethylphenyl)-10-(4-acetyl-3-hydroxy-2-propyl-phenoxy)-deca-3(E),5(Z)-diene-2yl-7-thio-4-oxo-4H-1-benzopyran-2-carboxylic-acid-sodium salt), CGP 57698 (4-[3-(7-fluoro-2-quinolinyl-methoxy)phenyl-amino]-2,2-diethyl-4-oxo-butanoic acid), ONO 1078 (4-oxo-8-[4-(4-phenylbutoxy)benzoylamino]-2-(tetrazol-5-yl)-4H-1-benzopyran hemihydrate) and ICI 204,219 (4(5-cyclopentyloxycarbonylamino-1-methylindol-3-yl-methyl)-3-methoxy-N-*O*-tolylsulphonyl-benzamide) were kindly provided by Dr A. von Sprecher (Ciba-Geigy Ltd., Basel, Switzerland). Cysteine, glycine, HEPES, acetylcholine, L-cysteine and mepyramine were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Filtercount was from Packard Instruments Company (Meriden, CT, U.S.A.). All the reagents used in h.p.l.c. analysis were of analytical grade and purchased from Carlo Erba (Milan, Italy) as were GF/C Whatman fibre glass filters. Liometacen (indomethacin) was from Chiesi Farmaceutici (Parma, Italy) and anti-IgE from KP laboratories Inc. (Gaithersburg, MD, U.S.A.). All salts for the Tyrode solution preparation were purchased from Merck (Darmstadt, Germany).

Results

Competition binding experiments

In all the experiments performed, [^{3}H]-LTD_4 mixed curves spanned more than two orders of magnitude, suggesting an interaction with two classes of binding sites. The simultaneous analysis of all LTD_4 control curves performed allowed the definition of the model with the affinities and the capacities indicated in Table 1.

Competition curves for iralukast and CGP 57698 (Figure 1) were performed by co-incubating the antagonists with [^{3}H]-LTD_4; the curves were monophasic, extending for two orders of magnitude, suggesting the interaction of each antagonist with a single class of binding sites (Table 2).

Effect of preincubation on antagonist binding vs [^{3}H]-LTD_4

Data previously obtained in guinea-pig lung membranes (von Sprecher *et al*., 1991a,b) indicated that preincubation increased iralukast affinity. For this reason, in human lung parenchyma we assessed the displacement of [^{3}H]-LTD_4 by a fixed concentration (0.1 μM) of iralukast, CGP 57698, ONO 1078 and ICI 204,219 with or without 15 min preincubation. Results presented in Figure 2 indicate that only CGP 57698 binding was basically unaffected by the preincubation time, while the displacement induced by the other antagonists was significantly higher ($P<0.01$) after preincubation. Furthermore, complete displacement curves were performed for iralukast and CGP 57698 preincubated for 15 min. Table 2 shows the effect of 15 min preincubation on K_i values for iralukast and CGP 57698 interaction with the low affinity state of the *CysLT* receptor, obtained in classical competition studies. The comparison of the ratios between K_i values obtained with and without preincubation indicates that iralukast affinity is markedly higher after preincubation, while, in agreement with the results of Figure 2, CGP 57698 affinity is unchanged. Thus, human lung membranes were routinely preincubated for 15 min with the antagonists in [^{3}H]-LTD_4 receptor binding assays.

Multiligand experiments

To assess the affinity of the antagonists for each of the two affinity states of the *CysLT* receptor, multiligand experiments were performed. To optimize the multiligand protocol, we simulated (MacSIMUL, G.E. Rovati and P.J. Munson) the theoretical model for the simultaneous interaction of an antagonist with two affinity states of the *CysLT* receptor (Figure 3). In particular, we hypothesized the interaction of an unselective receptor antagonist, with the binding characteristics of CGP 57698 (Table 2), with the high and low affinity

Table 1 Binding affinities and capacities for the receptor labelled by [^{3}H]-LTD_4 in human lung parenchyma

	K_d (nM ± % CV)	B_{max} (fmol mg^{-1} protein ± % CV)
High affinity binding site	0.054 ± 90	1.32 ± 87
Low affinity binding site	2.43 ± 83	30.5 ± 59

% CV = % coefficient of variation.

sites labelled by a selective agonist with the binding characteristics of LTD_4 (Table 1). The simulation was conceived with an extended multiligand protocol (see Methods), including the control [^{3}H]-LTD_4 mixed curve, a family of three multiligand [^{3}H]-LTD_4 curves, each in the presence of a fixed concentration (0.1 – 1 – 10 nM, respectively) of an antagonist, and the classical competition curve in which the labelled ligand was supposed to be 0.5 nM (Figure 3).

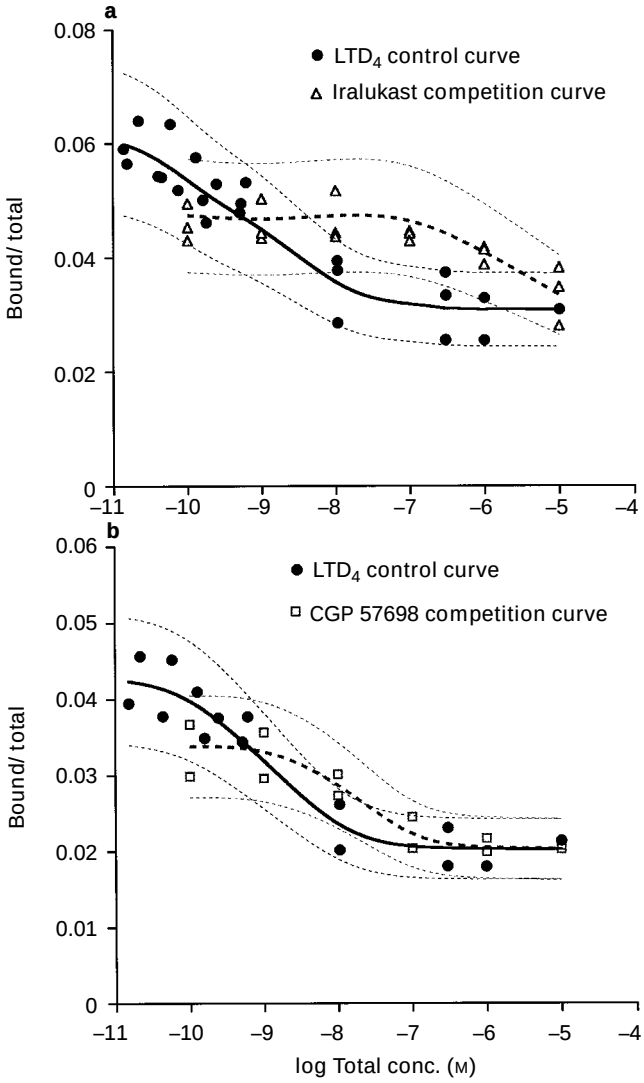

Figure 1 Mixed curves for [^{3}H]-LTD_4 and competition curves for iralukast (a) and CGP 57698 (b) vs [^{3}H]-LTD_4. Data are from three and two experiments for iralukast and CGP 57698, respectively, each performed in triplicate. Dotted lines represent ± 95% c.l.

Table 2 Effect of 15 min preincubation of human lung membranes on CGP 45715A (iralukast) and CGP 57698 affinities for the low affinity site of the receptor labelled by [^{3}H]-LTD_4

	K_i (nM ± % CV) *Preincubation* (min)		
	0	*15*	*0/15*
Iralukast	817 ± 36	11.4 ± 63	71.7
CGP 57698	7.6 ± 44	5.7 ± 32	1.3

% CV = % coefficient of variation.

Figure 4 shows the real binding curves obtained with the multiligand protocol for iralukast (a) and CGP 57698 (b) vs [^{3}H]-LTD_4. Furthermore, Figure 5 shows the binding curves obtained by use of the same type of experiment with ICI 204,219 (a) and ONO 1078 (b). Analysis of all the multiligand curves indicated that each antagonist was able to compete for both the sites labelled by [^{3}H]-LTD_4, without discriminating between them ($K_{i1} = K_{i2}$). Finally, Table 3 presents a summary of K_i values for the antagonist interaction with the *CysLT* receptor obtained from computer analysis.

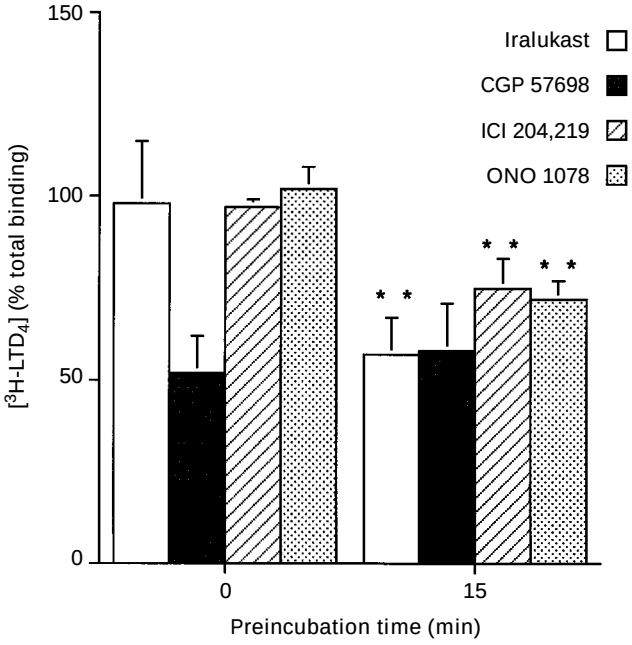

Figure 2 Effect of preincubation time on the inhibition of [^{3}H]-LTD_4 binding by a fixed concentration (0.1 μM) of antagonists. Columns represent the means ± s.e. of three experiments performed in duplicate. $^{**}P < 0.01$ vs 0 min preincubation time (two-way ANOVA).

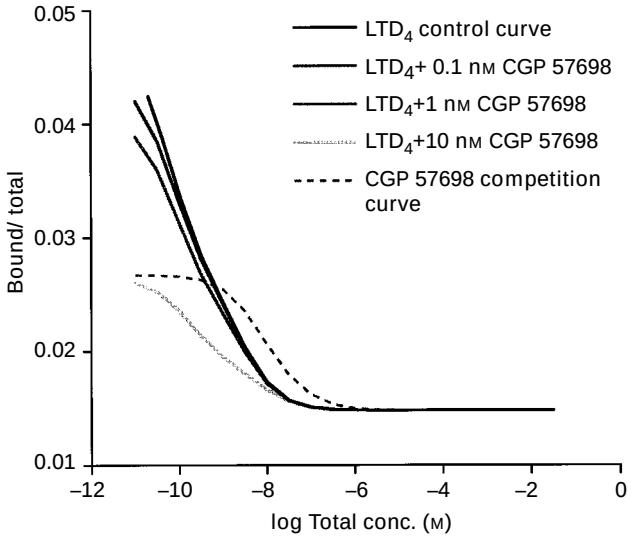

Figure 3 Theoretical model for the interaction of a non-selective receptor antagonist (i.e. CGP 57698, $K_{i1} = K_{i2} = 5.7$ nM) with the high and low affinity states of the receptor labelled by [^{3}H]-LTD_4 ($K_{d1} = 0.054$ nM and $K_{d2} = 2.43$ nM).

Antagonism of LTD_4-induced contraction of human isolated bronchial strips

Treatment with 3 mM L-cysteine did not significantly alter the basal tone of bronchial strips (data not shown). The addition of cumulative concentrations of LTD_4 (0.1 nM – 3 μM) induced a concentration-dependent contraction with an EC_{50} of 5.9 nM $\pm$ 7.5% coefficient of variation (%CV), reaching a maximum at approximately 1 μM in the control strip (Figure 6). The maximal response was 119.7 $\pm$ 1.3% CV of that elicited by 300 μM ACh.

Iralukast and CGP 57698 produced parallel rightward shifts in the cumulative LTD_4 concentration-response curves (Figure 6a and b, respectively). Moreover, the upper plateaux of the CGP 57698 curves were significantly higher ($P<0.01$) than that of the control curve. Figure 7 shows the Schild plots for iralukast and CGP 57698: the slopes of both the individual lines were not significantly different from 1 (slope = 0.99 $\pm$ 0.60, 95% confidence limits (95% c.l.) and 1.08 $\pm$ 0.88, 95% c.l., respectively) and the calculated pA_2 values were 7.77 $\pm$ 4.3% CV and 8.51 $\pm$ 1.6% CV, respectively.

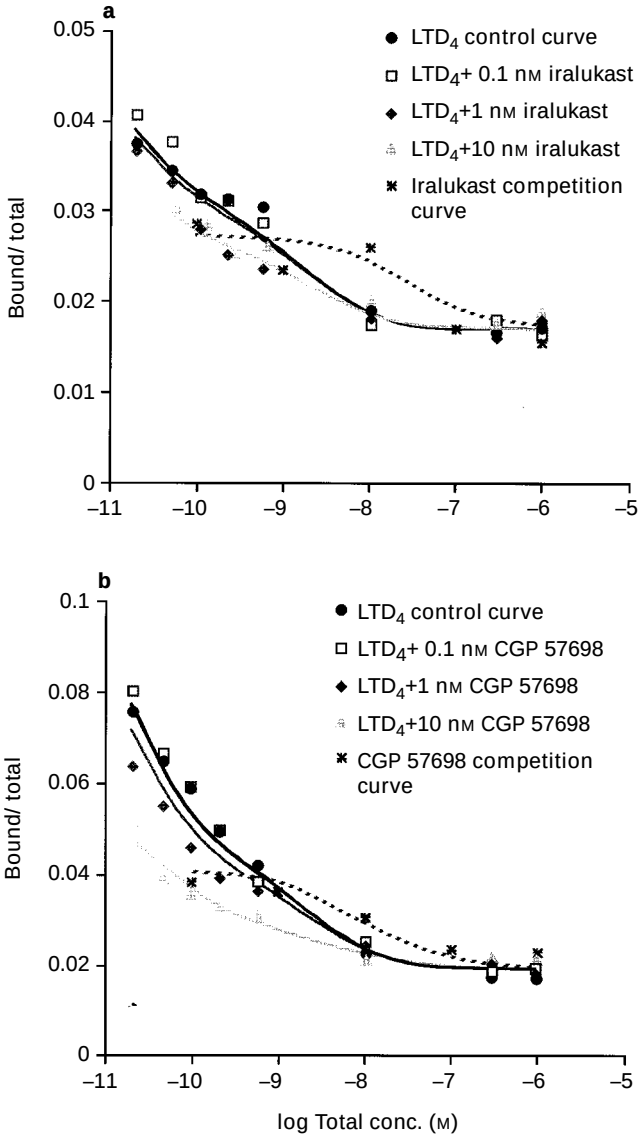

Figure 4 Multiligand curves for iralukast (a) and CGP 57698 (b) vs [^{3}H]-LTD_4. Data are means of three replicates from a single experiment, representative of at least two other experiments. LTD_4 control curve overlays the multiligand curve in the presence of 0.1 nM iralukast and CGP 57698.

Antagonism of antigen-induced contraction of human isolated bronchial strips

The addition of cumulative concentrations of anti-IgE (0.01 – 3.0 μg ml^{-1}) caused a progressive increase in bronchial tone, slow in onset and long-lasting, reaching a plateau at 1.0 –

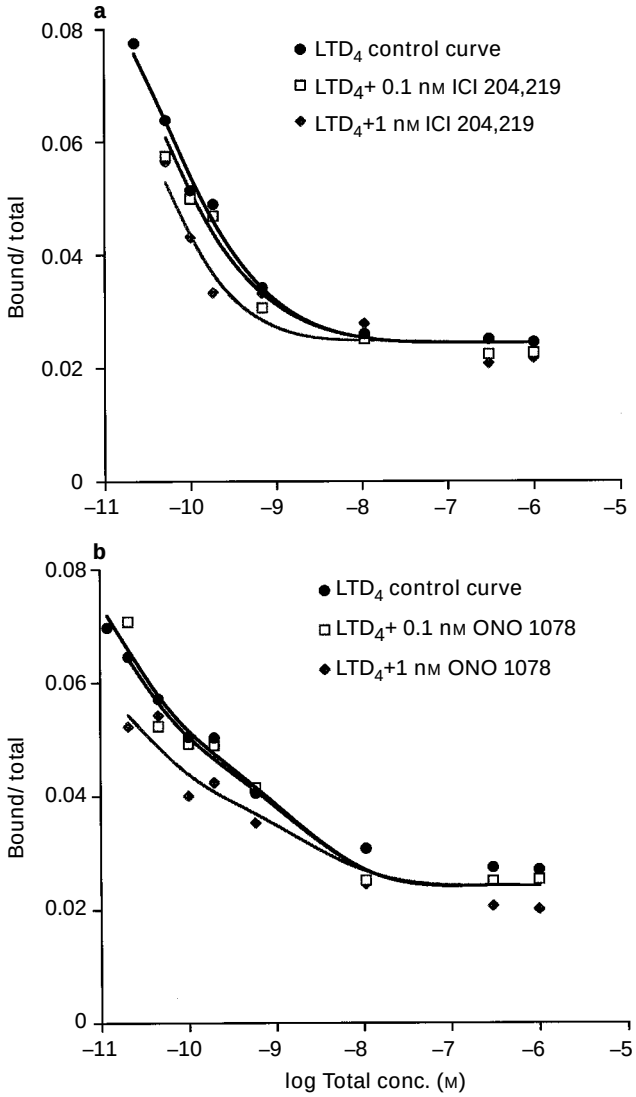

Figure 5 Multiligand curves for ICI 204,219 (a) and ONO 1078 (b) vs [^{3}H]-LTD_4. Data are means of three replicates from a single experiment, representative of at least two other experiments.

Table 3 Affinities of the different antagonist for the high and low affinity binding sites of the receptor labelled by [^{3}H]-LTD_4

Antagonist	$K_{i1} = K_{i2}$ (nM $\pm$ % CV)
Iralukast	16.6 $\pm$ 36
CGP 57698	5.7 $\pm$ 19
ICI 204,219	1.2 $\pm$ 34
ONO 1078	1.7 $\pm$ 43

% CV = % coefficient of variation.

3.0 μg ml^{-1} (Figure 8). The maximal contractile tone observed in control tissues was approximately 90–95% of the maximal contractile response observed with ACh (300 μM), significantly lower ($P<0.01$) than that of exogenous LTD_4 (compare control strips in Figures 8 and 6). Preincubation with the indicated concentrations of iralukast and CGP 57698 inhibited the antigen-induced contraction and lowered the upper plateau in a concentration-dependent way. Furthermore, CGP 57698 appeared to be 10–30 fold more potent than iralukast in preventing antigen-induced contraction of human bronchial strips (compare (a) and (b) in Figure 8).

Discussion

During the past, evidence has been obtained on the nature of the receptor for LTD_4 and its signal transduction system in animal and human tissues. This receptor belongs to the 'superfamily' of the seven transmembrane domain receptors and recent studies indicate a molecular mass of 45 and 55 kDa in guinea-pig lung membranes (Metters & Zamboni, 1993) and human lung membranes (Nicosia et al., 1995), respectively. The transduction mechanism of the receptor for LTD_4 involves at least two G proteins (Mong *et al.*, 1987; Crooke *et al.*, 1989; Sjölander *et al.*, 1990) and, as expected for a G-protein coupled receptor, two different classes of sites exist. These two sites represent two affinity states of the same receptor interconverted by guanosine 5′-triphosphate (GTP) and its analogues, as demonstrated in guinea-pig lung (Aharony *et al.*, 1987; O'Sullivan & Mong, 1989; Watanabe 1990).

In agreement with these findings, we have shown that in HLPM [^{3}H]-LTD_4 recognizes two classes of binding sites with

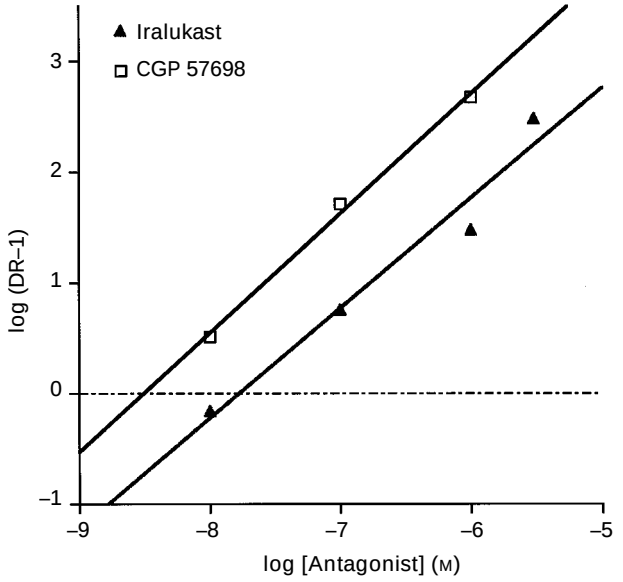

Figure 7 Schild plots for iralukast and CGP 57698. Data are from Figure 6.

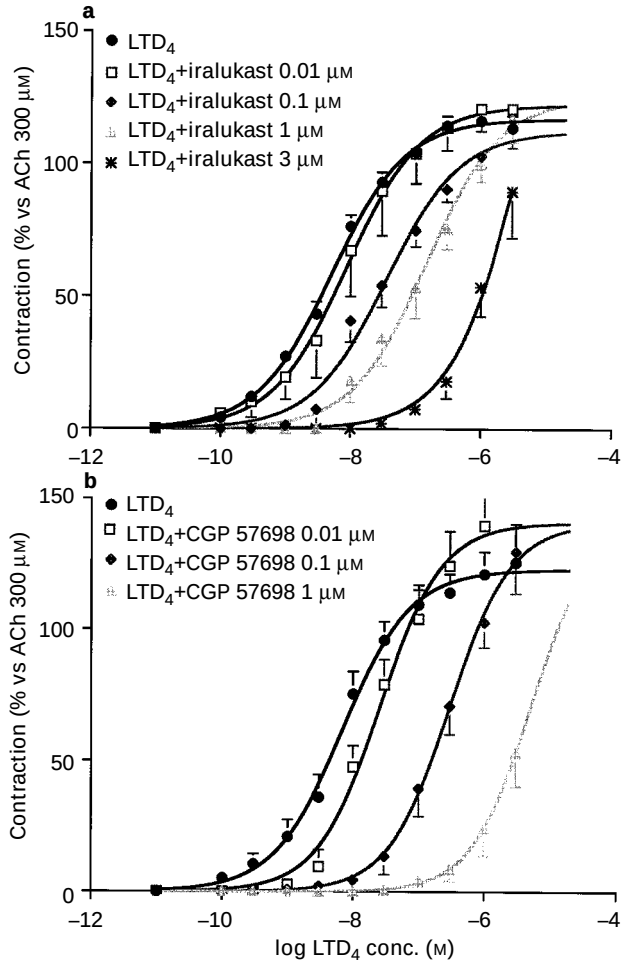

Figure 6 Effect of iralukast (a) and CGP 57698 (b) on LTD_4-induced contraction of human isolated bronchial strips. Each point is the mean of 4–6 replicates; vertical lines show s.e.mean.

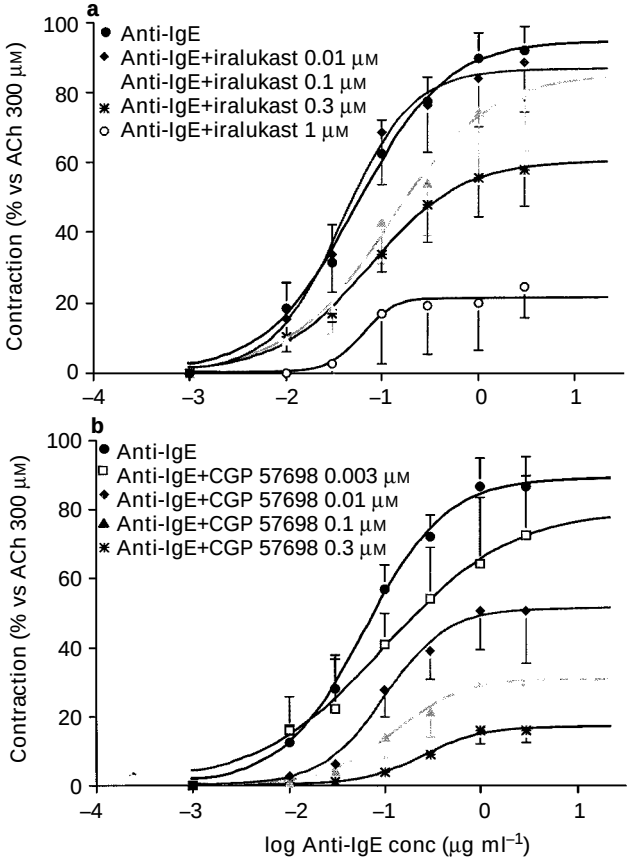

Figure 8 Effect of iralukast (a) and CGP 57698 (b) on anti-IgE-induced contraction of human isolated bronchial strips. Each point is the mean of 3–10 replicates; vertical lines show s.e.mean.

different affinities, and, as a consequence, the binding curves are biphasic (Figures 1, 4 and 5). The computer analysis of the mixed type curves confirmed the presence of two distinct binding sites for [^{3}H]-LTD_4, a high affinity-low capacity and a low affinity-high capacity binding site (Table 1). Furthermore, even in human lung parenchyma these sites can be interconverted by a GTP analogue (Capra *et al*., unpublished observations)

To characterize the two components of LTD_4 binding, it is necessary to perform binding curves with concentrations spanning several orders of magnitude within the same experiment. This has been achieved by performing the so called 'mixed type' binding curve (Rovati *et al*., 1991), analysed with the computer program LIGAND (see Methods). The absence of such an approach is probably why in a previous paper (Lewis *et al*., 1985) only one site was identified, with an affinity ($K_d = 0.15$ nM) intermediate between those we have identified (Table 1).

Binding studies previously performed on guinea-pig lung membranes (von Sprecher *et al*., 1991) demonstrated that preincubation of the tissue with iralukast resulted in an increase in the antagonist affinity, with the K_i value decreasing about one order of magnitude, suggesting a slow kinetic of binding. This increase in affinity is also reflected in displacement of [^{3}H]-LTD_4 binding on HLPM, where maximal inhibitory activity was achieved with preincubation times of 15 min (Figure 2), while a decrease in inhibitory potency was obtained at longer times (data not shown). In contrast, CGP 57698 affinity was unaffected by preincubation time, since the K_i values obtained with and without preincubation were not significantly different (Table 2). Moreover, the cysteinyl-LT antagonists in the most advanced development state, ICI 204,219 and ONO 1078 (Brooks & Summers, 1996), taken as reference compounds in binding studies, were also affected by preincubation (Figure 2) and, thus, [^{3}H]-LTD_4 binding was routinely performed after preincubating human lung membranes with the specific antagonist for 15 min.

We have performed a series of multiligand experiments in order to assess the affinity of the antagonists for each of the two affinity states of the *CysLT* receptor. In fact, by studying an unlabelled compound only with a classical heterologous competition curve one might incur in the so called 'self-displacement' by the labelled ligand (see Methods and Rovati, 1993).

Before performing the real multiligand experiments, we followed a theoretical approach assuming the antagonist to be: (1) able to interact with both classes of binding sites labelled by [^{3}H]-LTD_4; (2) a pure antagonist and therefore unable to distinguish between the high and low affinity state of a G-protein coupled receptor (De Lean *et al*., 1980; Lefkowitz *et al*., 1993).

The real multiligand experiments performed with all the antagonists indicate that the assumptions made in our simulation were correct. In fact: (1) the theoretical and the real curves were basically overlapping; (2) the analysis of the multiligand binding data confirmed that each antagonist interacted with both classes of binding sites labelled by [^{3}H]-LTD_4 and (3) that each antagonist was nonselective for the two sites labelled by [^{3}H]-LTD_4.

The results obtained from binding studies, summarized in Table 3, indicate that iralukast and CGP 57698 are high affinity ligands for the *CysLT* receptor on human lung membranes and that their affinities are similar to those observed for the reference compounds ICI 204,219 and ONO 1078.

The high affinity exhibited by iralukast and CGP 57698 in binding studies on human lung parenchyma seems to correlate well with the potency observed in functional studies performed in human bronchi, although caution should be taken in comparing different tissues. In fact, iralukast and CGP 57698 were able to cause a parallel rightward shift of the concentration-response curves for exogenous LTD_4-induced contraction of human isolated bronchi (Figure 6). ACh-induced contractions of the preparation were not affected by pretreatment with either antagonist (data not shown), confirming that the effect was specific for the *CysLT* receptor.

Although from the concentration-response curves CGP 57698 seems to be more potent than iralukast (Figure 6b and a, respectively), their calculated pA_2 values are not significantly different. The data plotted according to Schild show regression lines with slopes of 1.08 and 0.99 for CGP 57698 and iralukast, respectively, not significantly different from unity. Thus, statistical evaluation would suggest pure competitive antagonism. On the other hand, the upper plateaux representing the maximal responses in the presence of CGP 57698 were significantly higher than that of the control curve, even taking into account the minimal effect on baseline tone (5% drop) caused by the addition of either antagonist. Thus, the interaction of CGP 57698 at the *CysLT* receptor is surprisingly characterized by augmentative antagonism, as defined by the theory of Robertson and coworkers (Robertson *et al*., 1994) which assumes that receptors exist in two states, active and inactive, and that the augmentative antagonist has a lower affinity for the inactive state. This is the only theory that explains such antagonist behaviour, even if it is in partial disagreement with the most widely accepted theory on the two-state receptor model (Lefkowitz *et al*., 1993; Milligan 1995). As far as iralukast is concerned, no augmentative antagonism is apparent. However, in the guinea-pig tracheal and lung strip preparations, iralukast clearly exhibits an apparent non-competitive antagonism to LTD_4 challenge (Bray *et al*., 1990), whilst CGP 57698 is a full competitive antagonist of LTD_4-induced guinea-pig tracheal contractions (von Sprecher *et al*., 1996; 1997; and Bray, personal communication).

It is well known that immunological challenge of human bronchial airways is correlated with the release of histamine and cysteinyl-LTs (Dahlén *et al*., 1983). Since it is not easy to obtain pulmonary specimens from asthmatic subjects, we used a polyclonal antibody against human IgE to trigger a bronchoconstriction in the normal bronchial tissue, dependent on the mobilization of the endogenous precursor arachidonic acid and on cysteinyl-LTs (Viganò *et al*., 1990). The plateau occurring in control curves was probably due to saturation, by the anti-IgE antibody, of the IgEs present in the specimen, resulting in the maximal formation of endogenous cysteinyl-LTs that elicit a contraction significantly lower than that triggered by exogenous LTD_4 (compare Figures 6 and 8).

Our data demonstrate that iralukast and CGP 57698 are able to inhibit the allergen-induced contractions of human isolated bronchi, by significantly reducing the maximal response in a concentration-dependent manner; this might be a consequence of the above mentioned limited capacity to synthesize cysteinyl-LTs by mast-cells, present in the bronchial tissue, after antigen challenge. Thus, the addition of increasing concentrations of anti-IgE antibody cannot surmount the effect of the antagonists, which in any case act at a step downstream from the interaction of the stimulus with its target.

In conclusion, the results of the present *in vitro* investigation indicate that the compounds iralukast and CGP 57698 are potent *CysLT* receptor antagonists in human airways. Experiments simulating the pathological asthmatic attack confirm that these compounds might be useful tools in the therapy of asthma and other allergic diseases. Iralukast and

CGP 57698 display potencies in human tissues similar to the most advanced leukotriene antagonists that have recently been made available for clinical practice, and iralukast appears to be the longest-acting antagonist of its class in guinea-pig when given by the inhaled route (Bray *et al*., 1990).

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