ELSEVIER

Biochemical Pharmacology 63 (2002) 1537–1546

Biochemical Pharmacology

Pharmacological differences among $CysLT_1$ receptor antagonists with respect to LTC_4 and LTD_4 in human lung parenchyma

Saula Ravasi[a], Valérie Capra[a], Tiziana Panigalli[b], G. Enrico Rovati[a,*], Simonetta Nicosia[a]

[a]*Laboratory of Molecular Pharmacology, Department of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy*
[b]*Department of Clinical Surgery, San Paolo Hospital, University of Milan, Milan, Italy*

Received 18 September 2001; accepted 23 January 2002

Abstract

We have previously reported, by means of equilibrium binding studies, the existence of two distinct binding sites with receptor characteristics for LTC_4 and LTD_4 in human lung parenchyma (HLP) membranes using *S*-decyl-glutathione (*S*-decyl-GSH) to inhibit LTC_4 binding to a number of non-receptor sites. Recently, we have been able to avoid the use of *S*-decyl-GSH in kinetic experiments and to characterize a distinctive pharmacological profile for the LTC_4 high affinity binding sites which do not correlates with the ability of both LTD_4 and LTC_4 to contract isolated HLP strips through the $CysLT_1$ receptor. Here, we report that the most advanced $CysLT_1$ receptor antagonists, some of which are already in clinical use, displayed a different behavior toward LTC_4 and LTD_4 in HLP. Equilibrium and kinetic binding studies demonstrated the following rank order of potency for ^{3}H-LTD_4 receptor ($CysLT_1$): zafirlukast = montelukast > LM-1507 = LM-1484 = pranlukast. In addition, LM-1507, LM-1484, pranlukast and montelukast but not zafirlukast are able to interact also with the high affinity site for ^{3}H-LTC_4 (LM-1507 = LM-1484 > pranlukast; montelukast not detectable in the presence of *S*-decyl-GSH). In this respect, the behavior of the LM antagonists closely resembles that of pranlukast although LM-1507 and LM-1484 display a higher affinity for ^{3}H-LTC_4 sites. Montelukast has an intermediate behavior, inasmuch as its interaction with ^{3}H-LTC_4 sites can be revealed only in kinetic studies, while zafirlukast is totally unable to inhibit ^{3}H-LTC_4 binding. It might be, therefore, most relevant for a complete understanding of the clinical efficacy, besides their nominal potency, of the most advanced $CysLT_1$ receptor antagonists to consider their pharmacological differences with respect not only to LTD_4/LTE_4, but also to LTC_4.

Keywords: Cysteinyl-leukotriene; LM-1507; LM-1484; Montelukast; Human lung parenchyma; Asthma

1. Introduction

Cysteine-containing leukotrienes (Cys-LTs), LTC_4, LTD_4 and LTE_4, originate from the oxidative metabolism of arachidonic acid through a key enzyme, 5-lipoxygenase, in a number of inflammatory cells including eosinophils, basophils, mast cells and macrophages [1,2]. Recently, two different Cys-LT receptor have been cloned, namely $CysLT_1$ [3,4] and $CysLT_2$ [5–7], with a distribution that is clearly peculiar for each isoform. In particular, $CysLT_1$ seems more abundant in HLP, human bronchi and peripherial blood leukocytes and eosinophils [8], while $CysLT_2$ is more abundant in heart and brain [5]. The identification of the $CysLT_1$ receptor in the lung is consistent with the anti-bronchoconstrictive and anti-inflammatory actions of $CysLT_1$ receptor antagonists.

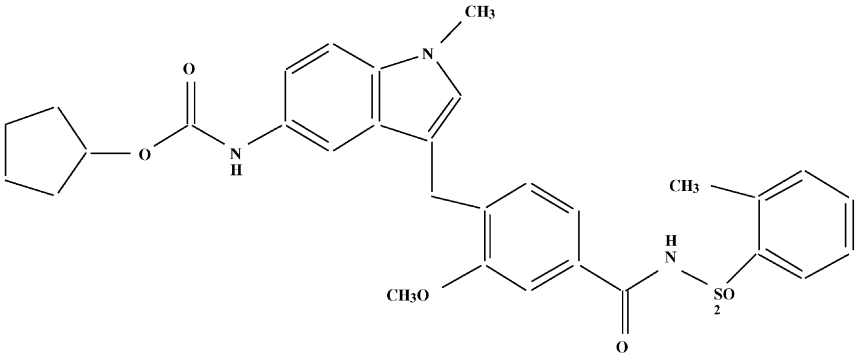
It is now widely recognized that Cys-LTs play an important role in asthma, participating both to the bronchoconstriction and to the chronic inflammatory component of the disease. Consequently, during the last years the number of structurally different $CysLT_1$ antagonists has been expanding at an ever increasing rate [9] (Fig. 1) and some are already available for clinical use (zafirlukast, montelukast and pranlukast) or in advance clinical trials (LM-1507, now MEN 91507). In particular, LM-1507 antagonizes in a potent and competitive manner LT-induced contraction in guinea pig ileum and human bronchi *in vitro*, while *in vivo* (administered by the i.v. or oral route) exerts a remarkable and long-lasting antagonism of LTD_4-induced bronchospatic and inflammatory responses.[1] However, all of them, to date, have been

* Corresponding author. Tel.: +39-2-5835-8369; fax: +39-2-5835-8385.
E-mail address: genrico.rovati@unimi.it (G.E. Rovati).

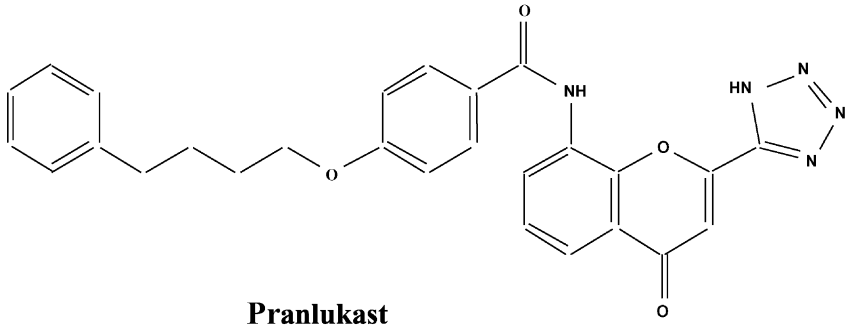
Abbreviations: LT, leukotriene; Cys-LT, cysteine-containing leukotrienes; *S*-decyl-GSH, *S*-decyl-glutathione; HLP, human lung parenchyma; RP-HPLC, reverse phase-high performance liquid chromatography.

[1] Dr. S. Manzini, Personal communications.

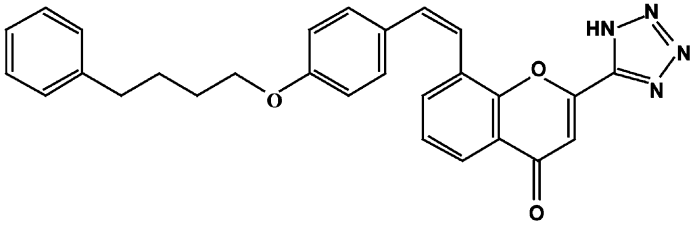
0006-2952/02/$ – see front matter
PII: S0006-2952(02)00889-4



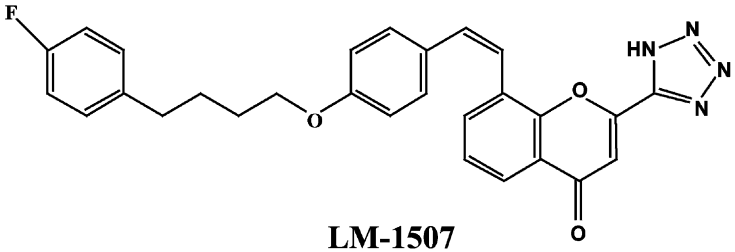
Zafirlukast



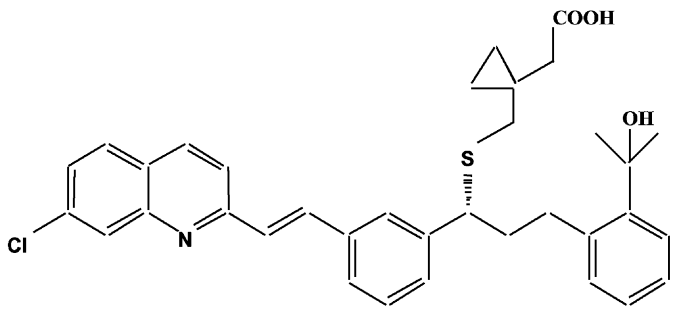
Pranlukast



LM-1484



LM-1507



Montelukast

Fig. 1. Chemical structure of $CysLT_1$ receptor antagonists.

designed to be $CysLT_1$ receptor antagonists, as it is generally believed that, in humans airways, LTC_4 acts through the same receptor as LTD_4 and LTE_4, namely the $CysLT_1$ receptor [10–12].

We recently pointed out, however, that in HLP membranes LTC_4 possess a specific high affinity binding site with characteristics distinct from those of LTD_4. In particular, in this tissue two of the classical $CysLT_1$ antagonists, i.e. pobilukast and ICI 198,615 (from which zafirlukast has been derived), have different selectivity with respect to ^{3}H-LTC_4 and ^{3}H-LTD_4 at equilibrium, thus suggesting that two different receptors might exist [13]. We also ruled out the hypothesis that this putative LTC_4 receptor might be a $CysLT_2$. In fact, BAY u9773, the only compound able to recognize both $CysLT_1$ and $CysLT_2$ receptors [14], was unable to dissociate ^{3}H-LTC_4 from its high affinity sites [15]. In addition, we have recently characterized a distinctive pharmacological profile for the LTC_4 high affinity binding site which does not correlate with the ability of both LTD_4 and LTC_4 to contract HLP. In fact, in functional experiments all the classical antagonists utilized were able to revert both LTC_4- and LTD_4-induced contractions of isolated HLP strips, thus indicating that both agonist were acting through the same $CysLT_1$ receptor [15].

It is intriguing to speculate that this putative receptor is implicated in aspects of the asthmatic syndrome different from bronchoconstriction, such as smooth muscle hyperplasia and proliferation or mucus secretion. Indeed, there are data in the literature indicating a proliferative role of Cys-LTs in human airway epithelial [16] or smooth muscle cells [17]. These data not only suggest LTC_4 as a more potent mitogenic stimulus than LTD_4 [16], but also indicate LTD_4 to be a weak agonist with a different pharmacological profile compared to the classical contractile function mediated by the $CysLT_1$ receptor. In addition, it has been recently reported that an inducible form of $CysLT_1$ receptor is present in vascular endothelial cells and that aspirin-triggered lipoxin A_4 specifically compete at this receptor site [18]. These data seems to suggest the presence of new sites for action and antagonism in the already complex scenario of inflammatory and anti-inflammatory mediators of asthma.

We think, therefore, that it might be relevant for the complete understanding of the clinical efficacy of the $CysLT_1$ receptor antagonists to characterize their pharmacological differences with respect not only to LTD_4/LTE_4, but also to LTC_4. We report here that the most advanced $CysLT_1$ receptor antagonists, which, to date, have been considered a pharmacologically homogeneous class of compounds, actually have a different pharmacological behavior in HLP membranes when challenged against LTC_4 and LTD_4. These data seem to confirm that an "atypical" *Cys-LT* receptor exists in human airways and that this receptor might possess a different spectrum of activity [17].

2. Materials and methods

2.1. Materials

^{3}H-LTC_4 (164–173 Ci mmol^{-1}) and ^{3}H-LTD_4 (164–173 Ci mmol^{-1}) were purchased from DuPont NEN. LTC_4 and LTD_4 were purchased from Cayman Chemical Co. Zafirlukast (ICI 204,219), pranlukast (ONO 1078), LM-1507 (now MEN 91507) and LM-1484 were from Menarini (LM-1507 is an amphiphilic chromone derivative with no chiral centres, manufactured by a linear synthesis of six steps and developed as a sodium salt); montelukast was from Merck. *S*-Decyl-GSH, cysteine, glycine, boric acid, serine, Hepes were purchased from Sigma Chemical Co. Filtercount was from Packard Instruments Company. All the reagents used in HPLC analysis were of analytical grade and purchased from Carlo Erba as were GF/C Whatman fiber–glass filters.

2.2. Preparation of HLP membranes

Crude membranes were prepared from macroscopically normal specimens, removed at thoracotomy for lung cancer as previously described [19]. Briefly, specimens were minced and homogenized at 4° in 10 mM Hepes buffer, pH 7.4 (1:24, w/v), centrifuged at 770 *g* for 10 min and the supernatant centrifuged at 27,000 *g* for 20 min. The pellet was resuspended, centrifuged under the same condition and finally resuspended in 5% of the homogenization volume. Membrane aliquots were frozen at −80° and stored for no longer than 3 months. Prior to use, serine–borate complex (40 mM, final concentration in the assay, prepared as an equimolar solution of serine and boric acid), cysteine (10 mM) and glycine (10 mM) were added to the membrane suspension to avoid Cys-LT metabolism.

2.3. Reverse phase-high performance liquid chromatography (RP-HPLC)

Labeled and unlabeled leukotriene purity was always assessed by RP-HPLC. Only leukotrienes with a purity grade higher or equal to 95% were utilized. The Beckman HPLC system was equipped with a 110B Solvent Delivery Module, ODS Ultrasphere C18 column and a Programmable Detector Module 166 set at 280 nm. Both labeled and unlabeled leukotrienes were eluted isocratically with a filtered and degassed mixture of CH_3OH:H_2O:CH_3COOH (65:35:0.02 v/v/v), adjusted at pH 5.8 with NH_4OH, at a flow rate of 1 mL min^{-1}. To check the purity of tritiated leukotrienes, fractions were collected every 30 s and the radioactivity profile assessed by liquid scintillation counting (Ultima Gold, Packard).

2.4. Binding studies

Equilibrium binding studies were performed at 25° for 60 min with 0.03–0.5 nM ^{3}H-LTD_4 or 40 min with

0.03–0.5 nM ^{3}H-LTC$_4$ and unlabeled homologous or heterologous ligands at the indicated concentrations. A multiligand protocol was followed [20,21]. Ten micromolar *S*-decyl-GSH was present only in the case of ^{3}H-LTC$_4$ equilibrium experiments. Time-courses were performed at 25° with 0.5 nM ^{3}H-LTC$_4$ or ^{3}H-LTD$_4$. Dissociation was induced by adding 1 mM unlabeled leukotriene (homologous dissociation) or 10 μM unlabeled antagonist (heterologous dissociation). In both equilibrium and kinetic studies HLP membranes (0.25 mg per sample), 10 mM HEPES-KOH pH 7.4, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ were added to the incubation mixture to achieve a final volume of 250 mL. All the experiments have been performed under control metabolic conditions (see Section 2.2). Unbound ligand was separated by rapid vacuum filtration (Brandel Cell Harvester) onto glass–fiber GF/C filters soaked in 2.5% polyvinylalchool and the filters were washed twice with 4 mL of HEPES buffer at 4°. Radioactivity was measured in a liquid scintillation counter (Filter Count, Packard).

2.5. Computer analysis

Analysis of equilibrium ligand binding data was performed by means of the computer program LIGAND [22] that allows simultaneous analysis of replicate experiments. A series of models of increasing complexity involving from one to three binding sites were considered. Analysis of binding data of homologous dissociation time-courses were performed using the program KINFIT II [23], which calculates association and dissociation rates (k_{on} and k_{off}) and the number of binding sites (B_{max}). Binding is expressed as specific bound concentration vs. time. Data from heterologous dissociation time-courses were analyzed using EXPFIT [24], which calculates the coefficients *C* (amount or percent bound), and *R* (the apparent rate constant for the specified antagonist). No direct calculation of k_{off} is possible for the heterologous dissociation time-courses. Biphasic dissociation time-courses represent interaction with a heterologous population of sites, where the fast dissociation

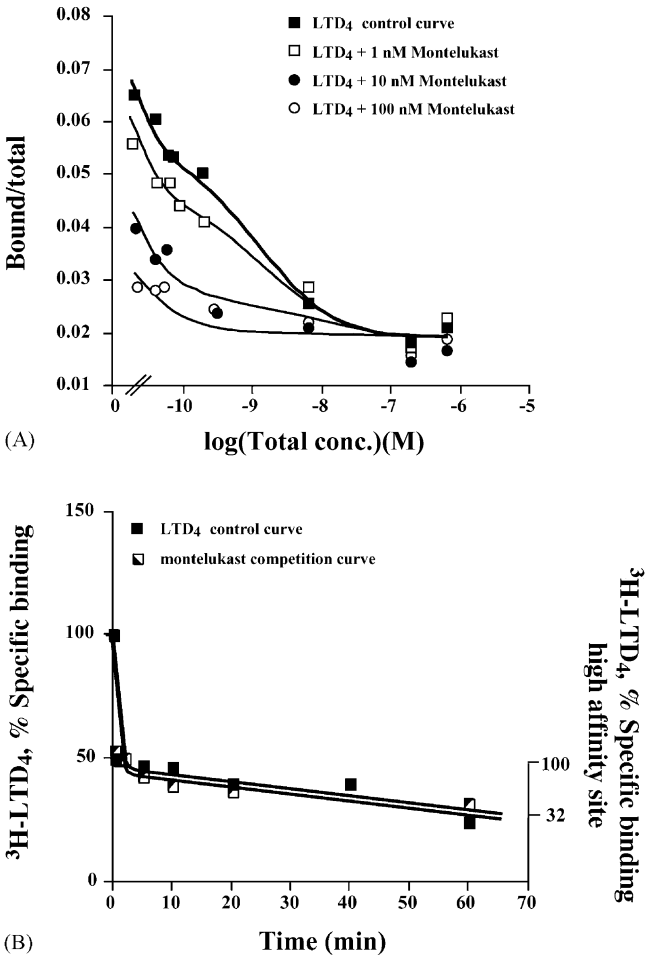


Fig. 2. (A) Equilibrium binding curves for ^{3}H-LTD$_4$ in the absence and presence of the indicated concentrations of montelukast (multiligand curves) ($n = 2$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTD$_4$ was induce by either 1 μM LTD$_4$ or 10 μM montelukast ($n = 3$). Right axis represents dissociation from the high affinity site only.

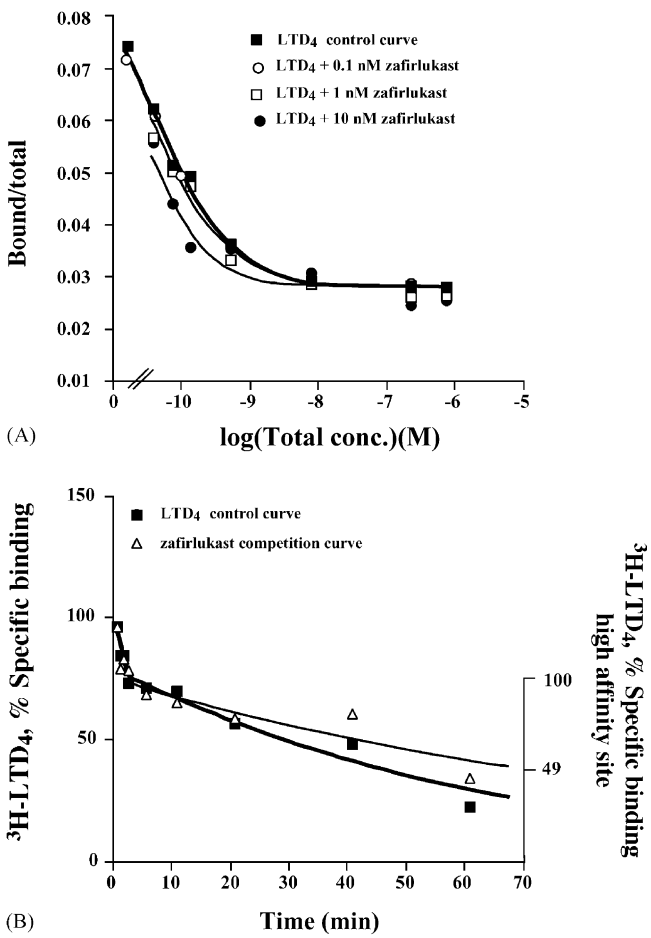


Fig. 3. (A) Equilibrium binding curves for ^{3}H-LTD$_4$ in the absence and presence of the indicated concentrations of zafirlukast (multiligand curves) ($n = 2$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTD$_4$ was induce by either 1 μM LTD$_4$ or 10 μM zafirlukast ($n = 3$). Right axis represents dissociation from the high affinity site only.

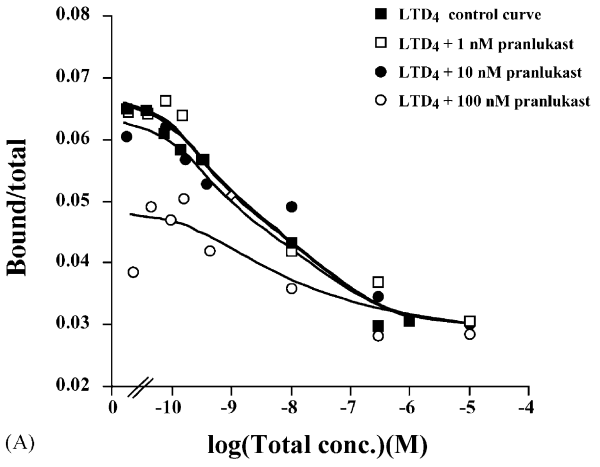
rate represents the low affinity component and the low dissociation rate represents the high affinity component. Antagonist competition is expressed as percentage dissociation specific binding. Different models of increasing complexity were selected using the statistical principle of the "extra sum of squares" [25]. Parameter errors are always expressed in percentage coefficient of variation (%CV). A statistical level of significance of $P < 0.05$ was accepted. All the curves shown were computer generated and represent a typical experiment.

3. Results

3.1. ^{3}H-LTD$_4$ binding

3.1.1. Equilibrium experiments

Figs. 2A–6A show the multiligand curves (i.e. curves of the labeled ligand in the absence and in the presence of a fixed concentration of the unlabeled ligand) for the CysLT$_1$ antagonists tested vs. ^{3}H-LTD$_4$, together with the "mixed curves" for ^{3}H-LTD$_4$ obtained within the same experiments. As expected, all computerized analysis of such experiments confirmed the presence of two classes of binding sites for ^{3}H-LTD$_4$ ($K_{d1} = 0.031$ nM ± 22%CV and $B_{max1} = 0.0034$ pmol mg^{-1} protein ± 50%CV; $K_{d2} = 3.8$ nM ± 66%CV and $B_{max2} = 0.13$ pmol mg^{-1} protein ± 60%CV), as predicted by the Ternary Complex Model [26] for agonist binding at a G-protein coupled receptor. On the contrary, all antagonists tested were better fitted by a single class model, again in agreement with Ternary Complex Model. Thus, all CysLT$_1$ antagonists tested recognize a single binding site in equilibrium studies with the following order of potency: zafirlukast = montelukast > LM-1507 = LM-1484 = pranlukast (montelukast vs. LM-1507, $P < 0.01$). K_i values are reported in Table 1.



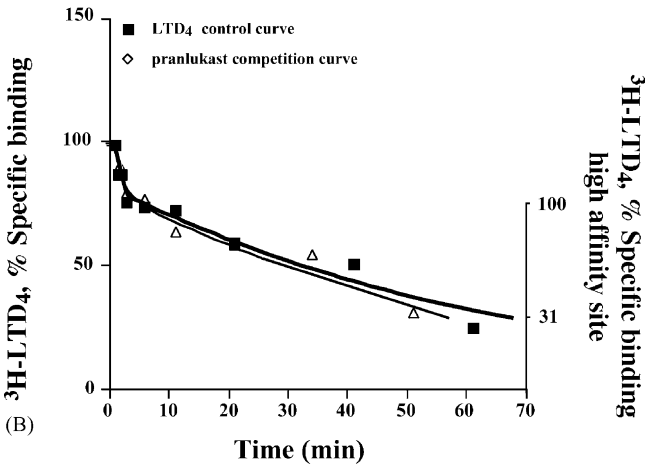
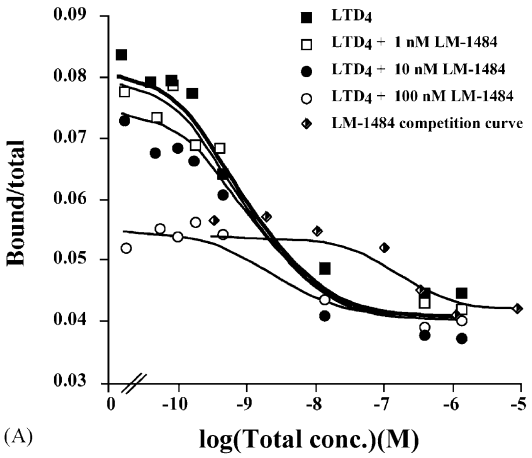


Fig. 4. (A) Equilibrium binding curves for ^{3}H-LTD$_4$ in the absence and presence of the indicated concentrations of pranlukast (multiligand curves) ($n = 2$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTD$_4$ was induce by either 1 μM LTD$_4$ or 10 μM pranlukast ($n = 3$). Right axis represents dissociation from the high affinity site only.



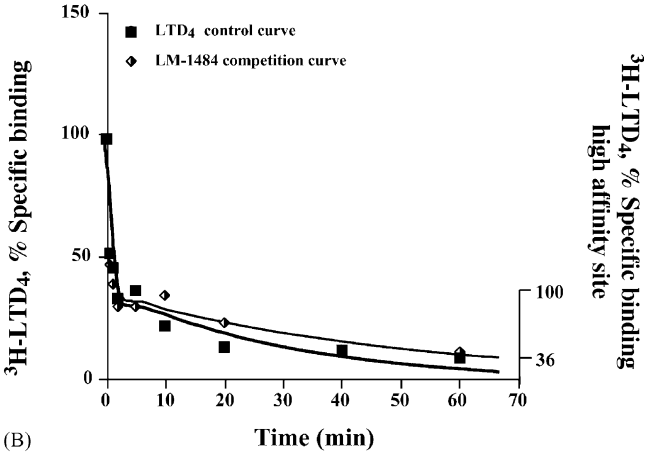


Fig. 5. (A) Equilibrium binding curves for ^{3}H-LTD$_4$ in the absence and presence of the indicated concentrations of LM-1484 (multiligand curves) ($n = 2$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTD$_4$ was induce by either 1 μM LTD$_4$ or 10 μM LM-1484 ($n = 3$). Right axis represents dissociation from the high affinity site only.

3.1.2. Kinetic experiments

In parallel with equilibrium binding studies, we also performed heterologous dissociation time-courses of

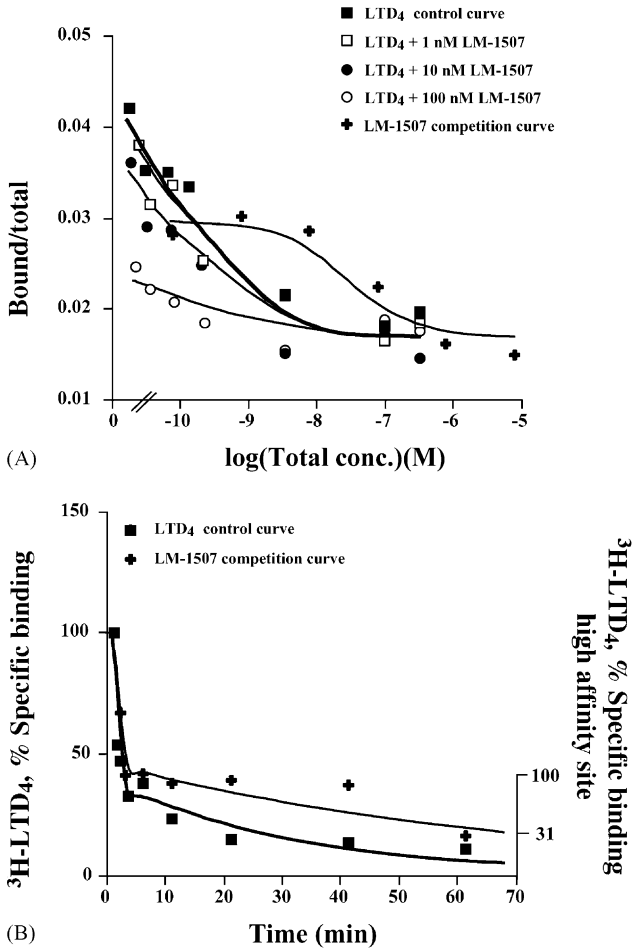


Fig. 6. (A) Equilibrium binding curves for ^{3}H-LTD$_4$ in the absence and presence of the indicated concentrations of LM-1507 (multiligand curves) ($n = 2$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTD$_4$ was induce by either 1 μM LTD$_4$ or 10 μM LM-1507 ($n = 3$). Right axis represents dissociation from the high affinity site only.

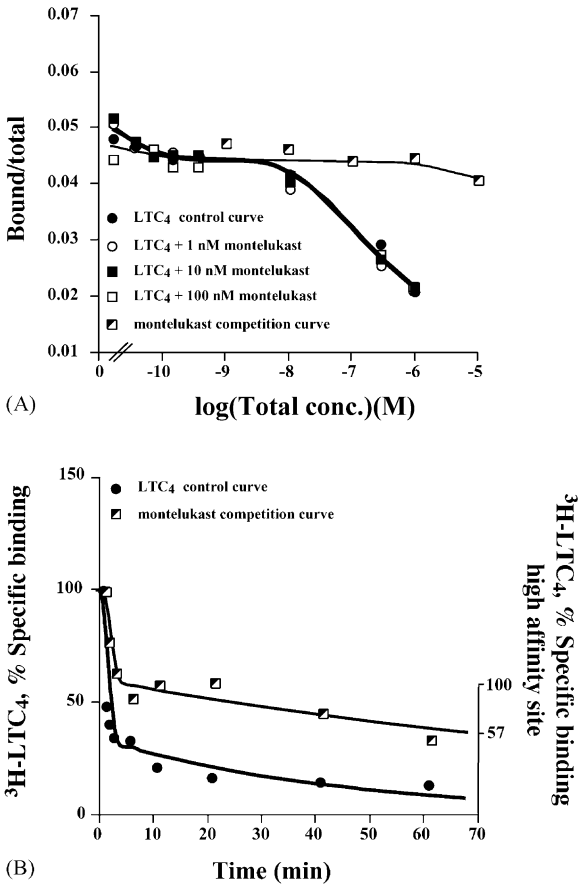


Fig. 7. (A) Equilibrium binding curves for ^{3}H-LTC$_4$ in the absence and presence of the indicated concentrations of montelukast (multiligand curves), and classical displacement of ^{3}H-LTC$_4$ by montelukast ($n = 3$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTC$_4$ was induce by either 1 μM LTC$_4$ or 10 μM montelukast ($n = 3$). Right axis represents dissociation from the high affinity site only.

CysLT$_1$ antagonists in comparison with a ^{3}H-LTD$_4$ homologous dissociation curve (Figs. 2B–6B). As expected, all the antagonists tested (Table 1) were able to dissociate ^{3}H-LTD$_4$ from its binding sites. In addition, both LTD$_4$- and antagonist-induced ^{3}H-LTD$_4$ dissociation curves were biphasic ($P < 0.05$), since these experiments represent the dissociation of the labelled ligand (^{3}H-LTD$_4$), induced by an unlabelled ligand (LTD$_4$ or one of the antagonists). The dissociation constant (k_{off1} and k_{off2}) for ^{3}H-LTD$_4$ are 1.7×10^{-4} s^{-1} ± 39%CV and 4×10^{-3} s^{-1} ± 45%CV, respectively. On the contrary, no direct calculation of k_{off} was possible for the heterologous dissociation time-courses. Table 1 only reports the percentage dissociation of the high affinity site at 60 min.

Table 1
Parameters of the different antagonists for the receptor labeled by ^{3}H-LTD$_4$ in human lung parenchyma

	Equilibrium experiments $K_{i1} = K_{i2}$ (nM ± %CV)	Kinetic experiments % dissociation at 60 min	
		High + low affinity sites ± % CV	High affinity site only
LM-1484	52 ± 25	88 ± 7	64
LM-1507	33 ± 41	84 ± 3	69
Pranlukast	84.7 ± 23	60 ± 10	69
Zafirlukast	1.2 ± 34	56 ± 7	51
Montelukast	2.8 ± 46	63 ± 4	68

Parameters are calculated by means of simultaneous analysis of at least two different experiments.

3.2. ^{3}H-LTC$_4$ binding

3.2.1. Equilibrium experiments

Figs. 7A–11A show the multiligand curves for the CysLT$_1$ antagonists tested vs. ^{3}H-LTC$_4$, together with the "mixed curves" for ^{3}H-LTC$_4$ obtained within the same experiments. Computerized analysis of these experiments confirmed the presence of two classes of binding sites for ^{3}H-LTC$_4$ ($K_{d1} = 0.21$ nM ± 66%CV and $B_{max1} = 0.0095$ pmol mg^{-1} protein ± 50%CV; $K_{d2} = 113$ nM ± 30%CV and $B_{max2} = 8.1$ pmol mg^{-1} protein ± 37%CV). In equilibrium

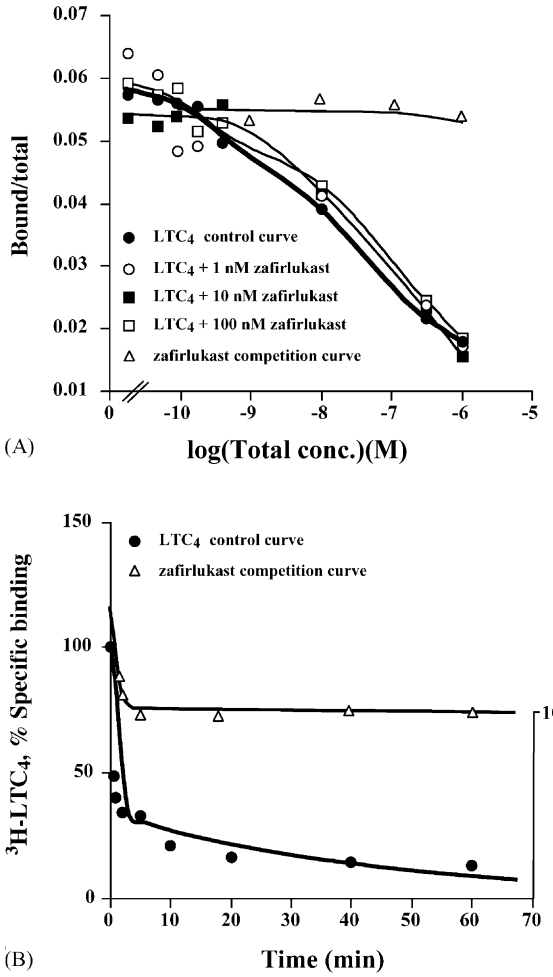


Fig. 8. (A) Equilibrium binding curves for ^{3}H-LTC$_4$ in the absence and presence of the indicated concentrations of zafirlukast (multiligand curves), and classical displacement of ^{3}H-LTC$_4$ by zafirlukast ($n = 3$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTC$_4$ was induce by either 1 μM LTC$_4$ or 10 μM zafirlukast ($n = 3$). Right axis represents dissociation from the high affinity site only.

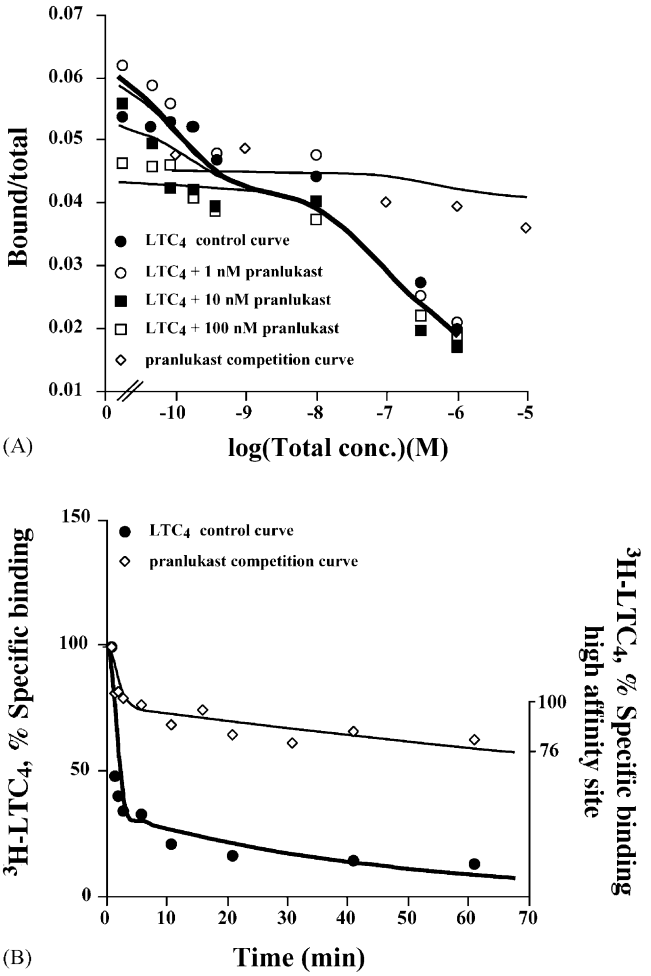


Fig. 9. (A) Equilibrium binding curves for ^{3}H-LTC$_4$ in the absence and presence of the indicated concentrations of pranlukast (multiligand curves), and classical displacement of ^{3}H-LTC$_4$ by pranlukast ($n = 3$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTC$_4$ was induce by either 1 μM LTC$_4$ or 10 μM pranlukast ($n = 3$). Right axis represents dissociation from the high affinity site only.

studies, however, only pranlukast, LM-1484 and LM-1507 (Figs. 9A–11A, respectively) were able to displace ^{3}H-LTC$_4$ from its binding sites. These antagonists recognize two classes of binding site, a high affinity one and a low affinity one. The rank order of potency vs. the high affinity class of sites in the presence of *S*-decyl-GSH is: LM-1507 = LM-1484 > pranlukast (LM-1484 vs. pranlukast, $P < 0.01$). K_i values are reported in Table 2. At equilibrium, zafirlukast was unable to compete with both the sites labeled by LTC$_4$ (Fig. 8A), while montelukast only marginally interacted with the low affinity one (Fig. 7A).

3.2.2. Kinetic experiments

Dissociation time-courses were performed using ^{3}H-LTC$_4$ as labeled ligand. Figs. 7B–11B presents the heterologous dissociation curves for CysLT$_1$ receptor antagonists vs. ^{3}H-LTC$_4$, together with the homologous dissociation curves for LTC$_4$ obtained within the same experiments. The dissociation constant (k_{off1} and k_{off2}) for ^{3}H-LTC$_4$ are $7.8 \times 10^{-4}\,s^{-1} \pm 33\%CV$ and $2.7 \times 10^{-2}\,s^{-1} \pm 35\%CV$, respectively.

LM-1507, LM-1484 and pranlukast displaced ^{3}H-LTC$_4$ also in kinetic studies from both high and low affinity sites

Table 2
Parameters of the different antagonists for the receptor labeled by ^{3}H-LTC$_4$ in human lung parenchyma

	Equilibrium experiments		Kinetic experiments
	K_{i1} (nM ± %CV)	K_{i2} (nM ± %CV)	% dissociation high affinity site at 60 min ± % CV
LM-1484	0.55 ± 73	12000 ± 31	40 ± 12
LM-1507	0.26 ± 68	17000 ± 24	38 ± 13
Pranlukast	32 ± 86	27000 ± 95	24 ± 4
Zafirlukast	N.D.	N.D.	N.D.
Montelukast	N.D.	24000 ± 59	43 ± 11

Parameters are calculated by means of simultaneous analysis of at least two different experiments.

N.D. = not detectable.

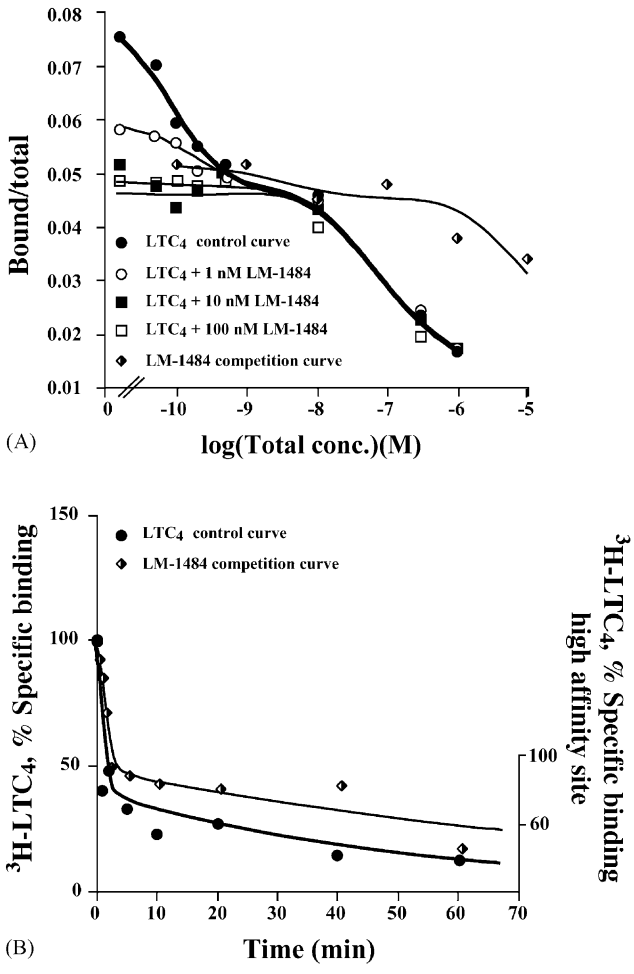


Fig. 10. (A) Equilibrium binding curves for ^{3}H-LTC$_4$ in the absence and presence of the indicated concentrations of LM-1484 (multiligand curves), and classical displacement of ^{3}H-LTC$_4$ by LM-1484 ($n = 3$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTC$_4$ was induce by either 1 μM LTC$_4$ or 10 μM LM-1484 ($n = 3$). Right axis represents dissociation from the high affinity site only.

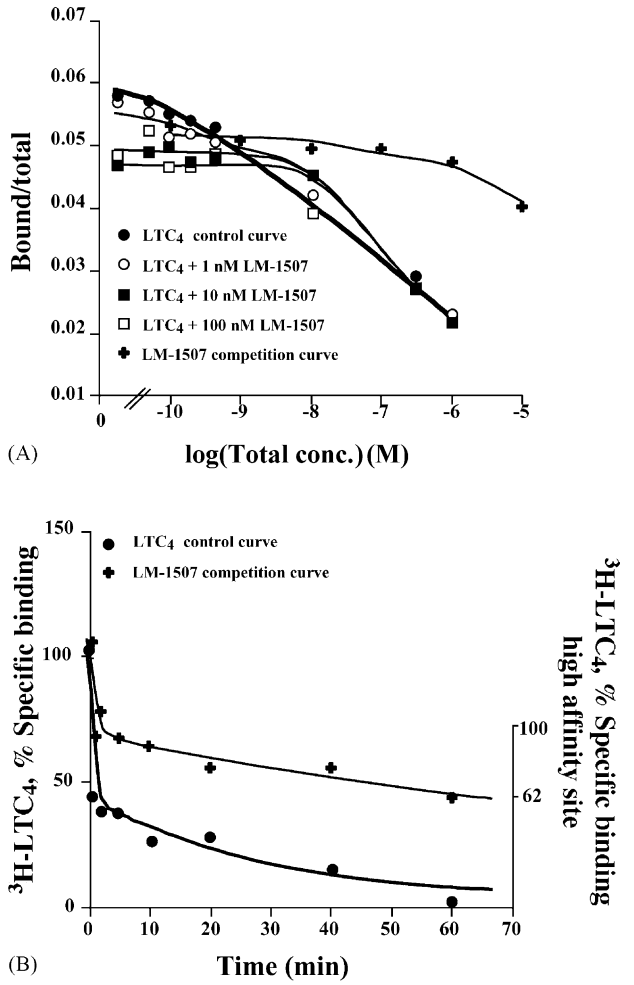


Fig. 11. (A) Equilibrium binding curves for ^{3}H-LTC$_4$ in the absence and presence of the indicated concentrations of LM-1507 (multiligand curves), and classical displacement of ^{3}H-LTC$_4$ by LM-1507 ($n = 3$). (B) Homologous and heterologous dissociation time-courses. Dissociation of ^{3}H-LTC$_4$ was induce by either 1 μM LTC$_4$ or 10 μM LM-1507 ($n = 3$). Right axis represents dissociation from the high affinity site only.

(Figs. 9B–11B and Table 2), confirming equilibrium data. However, with this experimental protocol also montelukast was able to interact with both sites labeled by ^{3}H-LTC$_4$ (Fig. 7B and Table 2). On the contrary, zafirlukast confirmed that it was unable to compete with ^{3}H-LTC$_4$ high affinity site, while only a minimal interaction with the low affinity site was detected (Fig. 8B).

4. Discussion

It is well known that LTC$_4$ predominantly binds to a number of non-receptor sites in cellular membranes [27,28] and, as we previously demonstrated, *S*-decyl-GSH, a ligand structurally similar to LTC$_4$ but devoid of either agonist or antagonist activities [29], must be routinely included in ^{3}H-LTC$_4$ binding assay at equilibrium to unmask a specific high affinity binding site for LTC$_4$ [13]. On the contrary, ^{3}H-LTD$_4$ binding is basically unaffected by the presence of *S*-decyl-GSH, as expected for a ligand with low affinity for enzymes such as glutathione-*S*-transferase. However, we also demonstrated that some antagonists were unable to compete for either ^{3}H-LTC$_4$ or ^{3}H-LTD$_4$ binding in the presence of *S*-decyl-GSH, suggesting that this compound interferes with antagonist but not with agonist binding [15]. A possible explanation for these findings could reside in a non total coincidence of agonist and antagonist sites on Cys-LT receptors and in the sterical hindrance of *S*-decyl-GSH at the antagonist binding site. Alternatively, recently Back *et al.* [30] have hypothesized that this might be due to formation of LTC$_4$ from LTD$_4$ in the presence of *S*-decyl-GSH. Thus, only LTC$_4$ binding was studied regardless of which agonist was added. This theory substantiates the presence of a different pharmacological profile for LTC$_4$ and LTD$_4$ in HLP membranes further supporting our hypothesis of a different LTC$_4$ receptor in this tissue.

We have also demonstrated that it is possible to avoid the use of *S*-decyl-GSH by performing heterologous dissocia-

tion time-courses, instead of equilibrium experiments, to study the pharmacological profile of the LTC_4 binding sites [15]. With kinetic experiments, one can perturb the equilibrium with the antagonists to assess their ability to dissociate ^{3}H-LTD_4 or ^{3}H-LTC_4 in the absence of *S*-decyl-GSH. The only limitation of this type of protocol is that no dissociation constants (k_{off}) or relative potency for the antagonists can be calculated, but only a qualitative analysis can be performed (see Tables 1 and 2). Thus, we have performed the pharmacological characterization of $CysLT_1$ receptor antagonists by means of both equilibrium and kinetic binding studies.

As expected, all the $CysLT_1$ receptor antagonists, i.e. zafirlukast, montelukast, LM-1507, LM-1484 and pranlukast, were able to displace ^{3}H-LTD_4 from its binding sites with different potencies both in equilibrium and in kinetic studies. In this respect, zafirlukast and montelukast have the lowest K_i values compared to the others compounds, in agreement with their known clinical potency [31]. Monophasic displacement curves in equilibrium studies suggest a lack of selectivity between the high and low affinity state of $CysLT_1$ receptor. Indeed, all the antagonists behave as ''pure'' antagonist with no inverse agonist activity detectable in this assay [32]. Biphasic dissociation time-courses are, however, expected also for nonselective ''pure'' antagonist, as in heterologous kinetic dissociation curves one actually plot the tracer kinetic (^{3}H-LTD_4, in this case) but not the unlabeled ligand kinetic (the antagonist). For the same reason, the dissociations of ^{3}H-LTD_4 from its high affinity site at a given time are all comparable.

On the contrary, only LM-1507, LM-1484 and pranlukast were able to compete with both ^{3}H-LTC_4 binding sites in equilibrium experiments, even in the presence of *S*-decyl-GSH. In this respect both the LM compounds have been demonstrated more potent than pranlukast in our experimental conditions. Neither montelukast nor zafirlukast showed any detectable interaction with either site. As mentioned before, the low affinity–high capacity site labeled by ^{3}H-LTC_4 in HLP membranes is of limited interest, inasmuch as it represents a mixture of nonreceptor site and, therefore, K_{i2} values reported in Table 2 have little or no significance. Thus, essentially, both zafirlukast and montelukast are not able to compete with the putative LTC_4 receptor in equilibrium studies. However, kinetic experiments performed in the absence of *S*-decyl-GSH revealed that montelukast is indeed able to dissociate ^{3}H-LTC_4 also from its high affinity receptor site, therefore, confirming that its inability to do so in equilibrium experiments is most likely due to the interference by *S*-decyl-GSH. On the contrary, zafirlukast did not show any detectable interaction with either site, confirming its inability to compete with ^{3}H-LTC_4. The kinetic studies confirmed the ability of LM-1507, LM-1484 and pranlukast to interact with both sites labelled by ^{3}H-LTC_4.

A number of $CysLT_1$ receptor antagonist, i.e. zafirlukast, but also iralukast and the dual $CysLT_1$–$CysLT_2$ antagonist BAY u9773 [15] do not seem to posses any significant antagonism vs. the sites labelled by ^{3}H-LTC_4. Interestingly, only LM-1507 and LM-1484 seem to have a high affinity for the putative LTC_4 receptor, while pranlukast appears to be between 60- and 120-fold less potent, respectively. Indeed, no K_i can be calculated for montelukast, and only functional studies will determine its rank order of potency in comparison to the other antagonist tested. Taken together, these binding data support our hypothesis that classical antagonists should no longer be considered a homogeneous class of compounds with respect to LTC_4 binding sites [15].

Moreover, these results confirm that HLP membranes contain two Cys-LT high-affinity binding sites with different pharmacological profiles. LTD_4 binding sites can be classified as a $CysLT_1$ receptor, while LTC_4 high affinity binding site is neither a $CysLT_1$ nor a $CysLT_2$ receptor. Although a direct evidence to correlate a specific physiological effect with the putative LTC_4 receptor is still lacking, our findings seem to confirm that an ''atypical'' *Cys-LT* receptor exists in human airways and that this receptor seems to possess a different spectrum of activity. In particular, it has been shown that LTD_4, besides its known contractile properties, is also able to augment epidermal growth factor-induced human airway smooth muscle cell proliferation. However, this additional effect has a peculiar sensitivity to $CysLT_1$ receptor antagonist, suggesting that the former phenomenon may be mediated by a Cys-LT receptor distinct from that which mediates LTD_4-induced human airway smooth muscle contraction [17]. It worth notice here that the pharmacological profile of this ''atypical'' new *Cys-LT* receptor suggested by these authors is indeed identical to the one proposed by us previously [15] and in this present report for LTC_4.

Many contractile agents [33,34] have been shown to induce proliferation of airway smooth muscle cells in culture and, thus, they might play an important role in stimulating the smooth muscle hypertrophy and hyperplasia associated with asthma. It must be remembered that all Cys-LT receptor antagonists up to date have been developed as specific and selective $CysLT_1$ receptor antagonists, a characteristic that might demonstrate not particularly convenient. Our finding of antagonist binding to a specific LTC_4 receptor, could contribute to the discovery and development of new and more active drugs with a wider spectrum of action to be used in the treatment of asthma.

Acknowledgments

The authors wish to acknowledge S. Manzini (Menarini, Firenze, Italy), P. Antonini (Merck Sharp & Dohme Italia, Rome, Italy) for providing LTD_4, LTC_4 and all the antagonists. The authors wish also to acknowledge the skillful contribution to this work of A. Tavola.

References

[1] Drazen JM, Austen KF. Leukotrienes and airway responses. Am Rev Respir Dis 1987;135:333–7.

[2] Hay D, Torphy TJ, Undem BJ. Cysteinyl leukotrienes in asthma: old mediators up to new tricks. Trends Pharmacol Sci 1995;16:304–9.

[3] Lynch KR, Gary P, O'neill GP, Qingyun Liu Q, Im D-S, Sawyer N, Metters KM, Coulombe N, Abramovitz M, Figueroa DJ, Zeng Z, Connolly BM, Bai C, Austin CP, Chateauneuf A, Stocco R, Greig GM, Kargman S, Hooks SB, Hosfield E, Williams Jr. DL, Ford-Hutchinson AW, Caskey CT, Evans JF. Characterization of the human cysteinyl leukotriene $CysLT_1$ receptor. Nature 1999;399: 789–93.

[4] Sarau HM, Ames RS, Chambers J, Ellis C, Elshourbagy N, Foley JJ, Schmidt DB, Muccitelli RM, Jenkins O, Murdock PR, Herrity NC, Halsey W, Sathe G, Muir AI, Nuthulaganti P, Dytko GM, Buckley PT, Wilson S, Bergsma DJ, Hay DW. Identification molecular cloning, expression and characterization of a cysteinyl leukotriene receptor. Mol Pharmacol 1999;56(3):657–63.

[5] Takasaki J, Kamohara M, Matsumoto M, Saito T, Sugimoto T, Ohishi T, Ishii H, Ota T, Nishikawa T, Kawai Y, Masuho Y, Isogai T, Suzuki Y, Sugano S, Furuichi K. The molecular characterization and tissue distribution of the human cysteinyl leukotriene CysLT(2) receptor. Biochem Biophys Res Commun 2000;274(2):316–22.

[6] Heise CE, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, Im D-S, Stocco R, Bellefeuille JN, Abramovitz M, Cheng R, Williams Jr. DL, Zeng Z, Liu Q, Ma L, Clements MK, Coulombe N, Liu Y, Austin CP, George SR, O'Neill GP, Metters KM, Lynch KR, Evans JF. Characterization of the human cysteinyl leukotriene 2 receptor. J Biol Chem 2000;275(39):30531–6.

[7] Nothacker H-P, Wang Z, Zhu Y, Reinscheid RK, Lin SHS, Civelli O. Molecular cloning and characterization of a second human cysteinyl leukotriene receptor: discovery of a subtype selective agonist. Mol Pharmacol 2000;58(6):1601–8.

[8] Figueroa DJ, Breyer RM, Defoe SK, Kargman S, Daugherty BL, Waldburger K, Liu Q, Clements M, Zeng Z, O'neill GP, Jones TR, Lynch KR, Austin CP, Evans JF. Expression of the cysteinyl leukotriene 1 receptor in normal human lung and peripheral blood leukocytes. Am J Respir Crit Care Med 2001;163(1):226–33.

[9] Claesson HE, Dahlen SE. Asthma and leukotrienes: antileukotrienes as novel anti-asthmatic drugs. J Int Med 1999;245(3):205–27.

[10] Buckner CK, Krell RD, Laravuso RB, Coursin DB, Bernstein PR, Will JA. Pharmacogical evidence that human intralobar airways do not contain different receptor that mediate contractions to leukotriene C_4 and leukotriene D_4. J Pharmacol Exp Ther 1986;237: 558–62.

[11] Hay DW, Muccitelli RM, Tucker SS, Vickery-Clark LM, Wilson KA, Gleason JG, Hall RF, Wasserman MA, Torphy TJ. Pharmacologic profile of SK&F 104353: a novel, potent and selective peptidoleukotriene receptor antagonist in guinea pig and human airways. J Pharmacol Exp Ther 1987;243(2):474–81.

[12] Buckner CK, Fedyna JS, Robertson JL, Will JA, England DM, Krell RD, Saban R. An examination of the influence of the epithelium on contractile responses to peptidoleukotrienes and blockade by ICI 204,219 in isolated guinea pig trachea and human intralobar airways. J Pharmacol Exp Ther 1990;252(1):77–85.

[13] Capra V, Nicosia S, Ragnini D, Mezzetti M, Keppler D, Rovati GE. Identification and characterization of two Cys-leukotriene high-affinity binding sites with receptor characteristics in human lung parenchyma. Mol Pharmacol 1998;53:750–8.

[14] Tudhope SR, Cuthbert NJ, Abram TS, Jennings MA, Maxey RJ, Thompson AM, Norman P, Gardiner PJ. BAY u9773, a novel antagonist of cysteinil-leukotrienes with activity against two receptor subtypes. Eur J Pharmacol 1994;264:317–23.

[15] Ravasi S, Capra V, Mezzetti M, Nicosia S, Rovati GE. A kinetic binding study to evaluate the pharmacological profile of a specific LTC_4 binding site not coupled to contraction in human lung parenchyma. Mol Pharmacol 2000;57:1182–9.

[16] Leikauf GD, Claesson HE, Doupnik CA, Hybbinette S, Grafstrom RC. Cysteinyl leukotrienes enhance growth of human airway epithelial cells. Am J Physiol 1990;259(4 Pt 1):255–61.

[17] Panettieri RA, Tan EM, Ciocca V, Luttmann MA, Leonard TB, Hay DW. Effects of LTD_4 on human airway smooth muscle cell proliferation, matrix expression, and contraction *in vitro*: differential sensitivity to cysteinyl leukotriene receptor antagonists. Am J Respir Cell Mol Biol 1998;19(3):453–61.

[18] Gronert K, Martinsson-Niskanen T, Ravasi S, Chiang N, Serhan CN. Selectivity of recombinant human leukotriene D(4) leukotriene B(4) and lipoxin A(4) receptors with aspirin-triggered 15-epi-LXA(4) and regulation of vascular and inflammatory responses. Am J Pathol 2001;158(1):3–9.

[19] Rovati GE, Oliva D, Sautebin L, Folco GC, Welton AF, Nicosia S. Identification of specific binding sites for leukotriene C_4 in membranes from human lung. Biochem Pharmacol 1985;34:2831–7.

[20] Rovati GE, Rodbard D, Munson PJ. DESIGN: computerized optimization of experimental design for estimating K_d and B_{max} in ligand binding experiments. (II. Simultaneous analysis of homologous and heterologous competition curves and analysis of blocking and of multiligand dose–response surfaces). Anal Biochem 1990;184:172–83.

[21] Rovati GE. Ligand-binding studies: old belief and new strategies. Trends Pharmacol Sci 1998;19(9):365–9.

[22] Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 1980; 107:220–39.

[23] Rovati GE, Shrager R, Nicosia S, Munson PJ. KINFIT II: a nonlinear least squares program for analysis of kinetic binding data. Mol Pharmacol 1996;50:86–95.

[24] Guardabasso V, Munson PJ, Rodbard D. EXPFIT: a program for simultaneous analysis of families of exponential decay curves. Comput Methods Progr Biomed 1988;27(1):55–63.

[25] Draper NR, Smith H. Applied regression analysis. New York: Wiley, 1966.

[26] De Lean A, Stadel JM, Lefkowitz RJ. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. J Biol Chem 1980;255:7108–17.

[27] Metters KM, Sawyer N, Nicholson DW. Microsomal glutathione *S*-transferase is the predominant leukotriene C_4 binding site in cellular membranes. J Biol Chem 1994;269(17):12816–23.

[28] Keppler D. Leukotrienes: biosynthesis transport inactivation and analysis. Rev Physiol Biochem Pharmacol 1992;121:1–30.

[29] Sala A, Civelli M, Oliva D, Spur B, Crea AEG, Folco GC, Nicosia S. Contractile and binding activities of structural analogues of LTC_4 in the longitudinal muscle of guinea-pig ileum. Eicosanoids 1990;3:105–10.

[30] Back M, Kumlin M, Cotgreave IA, Dahlen SE. An alternative pathway for metabolism of leukotriene D(4): effects on contractions to cysteinyl-leukotrienes in the guinea-pig trachea. Br J Pharmacol 2001;133(7):1134–44.

[31] Drazen JM, Israel E, O'Byrne PM. Treatment of asthma with drugs modifying the leukotriene pathway. N Engl J Med 1999;340(3):197–206.

[32] Costa T, Ogino Y, Munson PJ, Onaran HO, Rodbard D. Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. Mol Pharmacol 1992;41(3):549–60.

[33] Panettieri RA, Yadvish PA, Kelly AM, Rubinstein NA, Kotlikoff MI. Histamine stimulates proliferation of airway smooth muscle and induces c-fos expression. Am J Physiol 1990;259(6 Pt 1):L365–371.

[34] Panettieri Jr. RA, Goldie RG, Rigby PJ, Eszterhas AJ, Hay DW. Endothelin-1-induced potentiation of human airway smooth muscle proliferation: an ETA receptor-mediated phenomenon. Br J Pharmacol 1996;118(1):191–7.