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Pharmacological profile of MEN91507, a new $CysLT_1$ receptor antagonist

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Abstract

MEN91507 (8-[2-(*E*)-[4-[4-(4-fluorophenyl)butyloxy]phenyl]vinyl]-4-oxo-2-(5-1*H*-tetrazolyl)-4*H*-1-benzopyran sodium salt)) potently displaced [^{3}H]leukotriene D_4 binding from guinea-pig lung and dimethylsulphoxide-differentiated U937 (dU937) cell membranes (K_i 0.50 ± 0.16 and 0.65 ± 0.29 nM, respectively). On the other hand, MEN91507 did not display significant binding affinity for a series of receptors or channels. In functional studies on dU937 cells, MEN91507 behaved as insurmountable antagonist of leukotriene D_4-induced calcium transients, with an apparent pK_B of 10.25 ± 0.15. In anaesthetized guinea-pigs, MEN91507 antagonized in a dose-dependent manner leukotriene D_4-induced bronchoconstriction following i.v. or oral administration: the ED_{50s} were 3.0 ± 0.3 and 140 ± 90 nmol/kg, respectively. The inhibition of leukotriene D_4-induced bronchoconstriction by MEN91507 was long-lasting, since a dose of 0.6 μmol/kg produced 74% reduction of the response after 8 h from administration. Likewise, leukotriene D_4-induced microvascular leakage was antagonized by MEN91507 either following i.v. or oral administration: a significant inhibitory effect was still evident at 16 h from oral administration of a dose of 6 μmol/kg. It is concluded that MEN91507 is a potent and selective antagonist of both guinea-pig and human $CysLT_1$ receptors; in addition, in vivo studies on guinea-pigs indicate that MEN91507 is an orally available and long-lasting antagonist of the bronchomotor and pro-inflammatory effects induced by leukotriene D_4 through the stimulation of $CysLT_1$ receptors.

Keywords: Leukotriene receptor antagonist; CysLT receptor; Leukotriene D_4; Asthma; Bronchoconstriction; Airway inflammation

1. Introduction

Cysteinyl-leukotrienes, as leukotriene C_4, D_4 and E_4, are potent constrictors and pro-inflammatory mediators in guinea-pig and human airways. They play a crucial role in asthma pathophysiology by causing bronchoconstriction, mucus production, increase in vascular permeability and induce eosinophil-mediated inflammatory responses (Lane, 1998). Inhaled leukotriene D_4 and leukotriene E_4 provoke constriction of small and large airway smooth muscle and increase bronchial hyperresponsiveness to pharmacologic agents in both normal and asthmatic subjects. In addition, cysteinyl-leukotrienes has been identified in plasma, urine, nasal secretions, sputum and bronchoalveolar lavage fluid of patients during spontaneous exacerbations of asthma or after antigen challenge (Smith, 1998; Claesson and Dahlen, 1999). Although the initial focus was linked to broncho-constrictor activity, it is nowadays clear that the role of cysteinyl-leukotriene in asthma also involves a contribution to the underlying inflammation and in airway remodeling.

Cysteinyl-leukotrienes exert their biological actions by activating specific receptors on the membranes of target cells. Two types of CysLT receptors have been cloned and pharmacologically characterized, namely $CysLT_1$ and $CysLT_2$ (Gorenne et al., 1996; Rovati et al., 1997; Lynch et al., 1999; Sarau et al., 1999; Heise et al., 2000; Nothacker et al., 2000; Takasaki et al., 2000): both receptors belong to the superfamily of seven segments trasmembrane-spanning G-protein-coupled receptors. The $CysLT_1$ receptor appears to be

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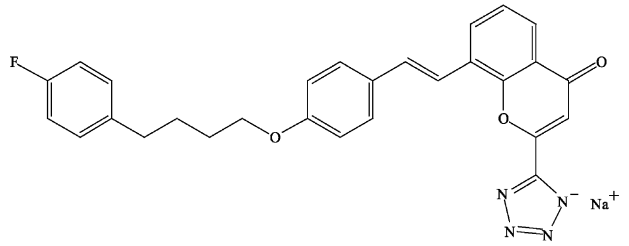

Fig. 1. Chemical structure of MEN91507.

the main responsible of the actions of cystenyl-leukotrienes in asthma (Lynch et al., 1999) and is specifically blocked by recently developed leukotriene D_4-receptor antagonists such as Montelukast (Jones et al., 1995), Zafirlukast (Krell et al., 1990), and Pranlukast (Obata et al., 1992). In contrast, the human $CysLT_2$ receptor is insensitive to these compounds (Heise et al., 2000; Nothacker et al., 2000).

Agents developed to specifically antagonize the actions of leukotriene D_4 or leukotriene E_4 at the receptor level, i.e., $CysLT_1$ receptor antagonists, represent a new class of drugs in therapy of asthma (Lipworth, 1999). Until now three $CysLT_1$ receptor antagonists, namely Montelukast, Pranlukast and Zafirlukast, have been developed and marketed (Hamilton et al., 1998; Leff et al., 1998; Nathan et al., 1998). They are orally effective over a wide range of asthma severity, preventing the airway inflammatory response to cysteinyl-leukotrienes action as well as acting as bronchodilators to the leukotriene-induced bronchoconstriction. Furthermore, recent clinical data would suggest that $CysLT_1$ receptor antagonists are also beneficial in upper airways diseases since Montelukast reduce symptoms in seasonal allergic rhinitis (Wilson et al., 2001).

This work describes the pharmacodynamic profile of the new $CysLT_1$ receptor antagonist MEN91507 (8-[2-(*E*)-[4-[4-(4-fluorophenyl)butyloxy]phenyl]vinyl]-4-oxo-2-(5-1*H*-tetrazolyl)-4*H*-1-benzopyran sodium salt)) (Fig. 1). The investigation has been conducted by means of in vitro studies and in animals models of bronchoconstriction and airway inflammation induced by leukotriene D_4. MEN91507 is a new potent, selective and orally effective $CysLT_1$ receptor antagonist: its preclinical profile indicates MEN91507 as a promising candidate for the treatment of inflammatory conditions of the respiratory tract, such as asthma and allergic rhinitis.

2. Methods

2.1. In vitro studies

2.1.1. Radioligand binding studies in guinea-pig lung membranes

Male Dunkin–Hartley guinea-pigs (250 to 400 g) were used as the source of lung membrane receptors. Membrane fractions containing CysLT receptors were prepared according to the method described by Mong et al. (1984) with minor modifications. Animals were killed by decapitation and the lung were removed and rinsed three times in Tyrode buffer (pH 7.4) and three times in phosphate-buffered saline (PBS). Lung tissue was minced and then homogenized in 250 mM sucrose, 20 mM Tris–HCl buffer (pH 7.4, at 4 °C) added with a cocktail of peptidase inhibitors: bacitracin (100 μg/ml), benzamidine (157 μg/ml), phenylmethyl–sulphonyl fluoride (87 μg/ml) and soybean trypsin inhibitor (100 μg/ml). The homogenate was centrifuged at 15 000 × *g* for 30 min at 4 °C. The supernatant was separated by filtration, centrifuged at 40 000 × *g* for 30 min at 4 °C and the pellet resuspended in homogenization buffer. Several aliquots were prepared, centrifuged again at 40 000 × *g* for 30 min at 4 °C and stored at −80 °C until use. Before binding experiments, aliquots were thawed with the corresponding buffer and protein concentration was determined by the method of Bradford (1976). Binding assays were performed in the following buffers: for [^{3}H]leukotriene D_4 and [^{3}H]leukotriene E_4 binding, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) buffer (pH 7.5) containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, 5 mM cysteine and 5 mM glycine; for [^{3}H]leukotriene C_4 binding, 10 mM Tris–HCl buffer (pH 7.4) containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, 5 mM cysteine, 5 mM glycine and 80 mM L-serine borate complex that inhibits metabolism of leukotriene C_4 to leukotriene D_4 by γ-glutamyltranspeptidase activity; and for [^{3}H]leukotriene B_4 binding, 10 mM Tris–HCl buffer (pH 7.4) containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$.

In the drug competition assays, incubation mixtures (0.31 ml) containing radiolabeled leukotriene ([^{3}H]leukotriene D_4, [^{3}H]leukotriene C_4 and [^{3}H]leukotriene B_4 0.5 nM, and [^{3}H]leukotriene E_4 1 nM), membrane protein (150 μg/ml for [^{3}H]leukotriene D_4, [^{3}H]leukotriene E_4 and [^{3}H]leukotriene B_4 binding, and 100 μg/ml for [^{3}H]leukotriene C_4 binding) and different concentrations of competing agents (agonist, antagonist or vehicle) were incubated at 25 °C for 30 min. The specific binding was defined as the difference between the amount of radiolabeled leukotriene bound in the absence and presence of cold leukotriene (1 μM leukotriene D_4, 4 μM leukotriene E_4, 5 μM leukotriene C_4 and 1 μM leukotriene B_4). In order to determine dissociation constants (K_d), the saturation binding experiments were performed by incubating an aliquot of guinea-pig lung membranes as above in the presence of increasing concentrations of labelled leukotriene (0.05 to 10 nM, or to 500 nM in the case of leukotriene C_4). The specific binding was defined as the difference between the amount of [^{3}H]leukotriene bound in the absence and in the presence of 1 μM non-labelled leukotriene. Binding reactions were stopped by filtration through Whatman GF/B glass microfiber filters under reduced pressure using a Brandel Cell Harvester. Filters were washed four times with 4 ml of ice-cold 10 mM Tris–HCl (pH 7.4) and 100 mM NaCl for [^{3}H]leukotriene D_4, and [^{3}H]leukotriene C_4; 10 mM Tris–HCl (pH 7.4) for [^{3}H]leukotriene E_4 and [^{3}H]leukotriene B_4. The

radioactivity retained on rinsed filters was determined by a liquid scintillation counter. Data from drug competition experiments were analyzed by a non-linear least-square regression analysis using programs of Equilibrium Binding Data Analysis (EBDA) software (McPherson, Elsevier-BIOSOFT, 1983) for simple analysis of competitive binding and LIGAND software (Munson and Rodbard, 1980) for saturation experiments.

2.1.2. Radioligand binding assay of [^{3}H]leukotriene D_4/leukotriene C_4 in dimethylsulphoxide (DMSO)-differentiated U937 (dU937) cell membranes

Human tumoral cell line, U937 (obtained from the American Type Culture Collection) were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 20 U/ml penicillin and 20 μg/ml streptomycin. The cells were cultured in 175 cm^2 tissue culture flasks and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Differentiation toward the macrophage-like state was induced by suspending 2×10^5 cells/ml in the same medium containing 1.3% dimethylsulphoxide (DMSO) for at least 4 days. DMSO-differentiated U937 (dU937) cell membranes and leukotriene receptor binding assays were performed according to the procedure described by Frey et al. (1993) with minor modifications. DU937 cells were harvested by centrifugation at $600 \times g$ for 10 min and the resulting cell pellet was resuspended in 35 ml cold PBS, pH 7.4, without Ca^{2+} and Mg^{2+} (PBS^-) and recentrifuged at $600 \times g$ for 10 min at 4 °C. All subsequent procedures were performed at 4 °C. The pellet was then resuspended at a cell concentration of 10^8 cells/ml with PBS^-, dispersed by gentle homogenisation and stored at −80 °C. These cell preparations were sonicated (four pulses of 30 s, $v = 70$, 4 °C) in the presence of phenylmethyl–sulphonyl fluoride (2 mM) and centrifuged at $10\,000 \times g$ for 10 min. The resulting supernatant was subjected to a final centrifugation at $100\,000 \times g$ for 30 min, resuspended at an equivalent of 10^9 cells/ml and stored at −80 °C until use. Binding assays were performed in a final volume of 500 ml of 10 mM HEPES/KOH, pH 7.4, containing 20 mM $CaCl_2$, 0.2 nM [^{3}H]leukotriene D_4 (146 Ci/mmol) and 20 mM of the peptidase inhibitor L-penicillamine. Non-specific binding was determined in the presence of 1 μM leukotriene D_4. For the leukotriene C_4 binding assay incubations included 20 mM $CaCl_2$, 0.5 nM [^{3}H]leukotriene C_4 (146 Ci/mmol) and 80 mM L-serine–borate complex. Non-specific binding was determined in the presence of 5 μM leukotriene C_4. The assay incubation also contained 70 μg of cell membrane protein. Incubations were conducted for 60 min at room temperature prior to separation of bound and free radiolabelled leukotriene by rapid filtration as described above.

The saturation binding experiments were performed by incubating an aliquot of cell membrane protein (70 μg) with various concentrations of [^{3}H]-leukotriene (0.05 to 10 nM). The specific binding was defined as the difference between the amount of [^{3}H] leukotriene bound in the absence and in the presence of cold leukotriene (1 μM leukotriene D_4 and 5 μM leukotriene C_4). Computational methods were as described in above radioligand bindings.

2.1.3. Study in various receptor binding assays

The selectivity of MEN91507 as CysLT receptor antagonist has been established by assessing its affinity for various receptors in radioligand binding assays. This study was conducted according to the established methods at Cerep (le Bois l'Eveque BP, 186600 Celle l'Evescault, France). Briefly, the displacement induced by MEN91507 (1 μM) was evaluated on human recombinant adenosine A_1 (radioligand ^{3}[H]DPCPX, 1 nM), A_{2a} (radioligand [^{3}H]CGS 21680, 6 nM), A_{2b} (radioligand [^{3}H]DPCPX, 5 nM), adrenergic α_{2A} (radioligand [^{3}H]RX 821002, 1.5 nM), α_{2C} (radioligand [^{3}H]RX 821002, 5 nM), β_1 (radioligand [^{3}H]CGP 12177, 0.15 nM), β_2 (radioligand [^{3}H]CGP 12177, 0.15 nM), bradykinin B_2 (radioligand [^{3}H]bradykinin, 0.2 nM), endothelin ET_A (radioligand [^{125}I]endothelin-1, 30 pM), endothelin ET_B (radioligand [^{125}I]endothelin-1, 10 pM), muscarinic M_1 (radioligand [^{3}H]pirenzepine, 2 nM), muscarinic M_3 ([^{3}H]4-DAMP, 0.2 nM), tachykinin NK_1 (radioligand [^{3}H][Sar^9, Met(O$_2$)^{11}]-SP, 0.5 nM), NK_2 (radioligand [^{125}I]NKA, 0.1 nM), serotonin 5-HT_{2A} (radioligand [^{3}H]ketanserin, 2 nM), 5-HT_{2C} receptors (radioligand [^{3}H]mesulergine, 0.7 nM), human myeloma cell IL_6 receptors (radioligand [^{125}I]interleukin-6, 50 pM), human neuroblastoma cell Y_2 receptors (radioligand [^{125}I]neuropeptide PYY, 15 pM), human platelet prostanoid IP (radioligand [^{3}H]iloprost, 10 nM), and TP receptors (radioligand [^{3}H]SQ 29548, 5 nM), rabbit platelet PAF receptors (radioligand [^{3}H]C_{16}-PAF, 1 nM), rat brain non-selective GABA (radioligand [^{3}H]GABA, 10 nM), glutamate NMDA (radioligand [^{3}H]CGP 39653, 5 nM), non-selective opioid (radioligand [^{3}H]naloxone, 1 nM), non-selective serotonin (radioligand [^{3}H]5-HT, 2 nM), Ca^{2+} channel receptors (radioligand [^{3}H](+)PN 200-110, 40 pM), guinea-pig lung histamine H_1 (radioligand [^{3}H]pyrilamine, 1 nM), striatal H_2 receptors (radioligand [^{125}I]APT, 0.1 nM), rat brain H_3 receptors (radioligand [^{3}H](*R*)α-metyl-histamine, 0.5 nM), and mouse fibroblast glucocorticoid receptors (radioligand [^{3}H]triamcinolone acetonide, 50 μM). Each determination was carried out in two separate experiments performed in duplicate.

2.1.4. Leukotriene D_4-induced $[Ca^{2+}]_i$ mobilization in dU937 cells

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was estimated using the calcium fluorescent probe, fura-2 (Grynkiewicz et al., 1985). Cells were washed twice with Hanks' Balanced Salt Solution containing 0.1% bovine serum albumin, resuspended in the same buffer at a concentration of 6×10^6 cells/ml and incubated with 2 μM fura-2 pentaacetoxymethyl ester (fura-2-AM), at 37 °C for 45 min. The cells were then centrifuged at 600 rpm for 5 min at room temperature and the pellet resuspended in fresh buffer and

incubated at 25 °C for an additional 30 min to allow complete hydrolysis of the intracellular fura-2 ester. DU937 cells were then washed twice, resuspended at a final concentration of 3×10^6 cells/ml and stored at 25 °C until used for fluorescence determination. A 2-ml sample of the cell suspension containing $6-7 \times 10^5$ total cells was added to a cuvette and placed into a Perkin-Elmer LS-50B spectrofluorimeter equipped with a warming block at 37 °C and stirring apparatus for constant mixing of the cell suspension. The wavelength of the spectrofluorimeter was set at 340 and 380 nm for excitation and 509 nm for emission. Fluorescence was monitored at the two wavelengths and $[Ca^{2+}]_i$ was calculated using a computer program (software WinLab from Perkin-Elmer) based on the equation of Grynkiewicz et al. (1985). Calibration was performed by adding ionomycin (20 μM) and then an excess of EGTA (22 mM). Cells were kept at 37 °C with continuous stirring for 2 min before $[Ca^{2+}]_i$ measurement. After the reaching of a stable baseline, leukotriene D_4 was added to the cell suspension and changes in $[Ca^{2+}]_i$ were monitored for an additional 3–4 min. The antagonists were preincubated for 2.5 min before the addition of leukotriene D_4. Concentration–response curves to leukotriene D_4, in the absence or in the presence of antagonists, were normalized towards the maximal response (E_{max}) reached by leukotriene D_4 (100–300 nM) and EC_{50} calculated.

2.2. In vivo studies

2.2.1. Animals

Male Dunkin–Hartley guinea-pigs, weighing 250 to 400 g, were used throughout the in vivo experiments. Animals were housed in a temperature and humidity-controlled room (12-h light and 12-h dark cycle) with free access to food and water. All animals used in the studies were cared for accordance with the principles and guidelines of the Local Government and regulations of European Union.

2.2.2. Measurement of leukotriene D_4-induced bronchoconstriction in anaesthetized guinea-pigs

Anti-bronchoconstriction activity of CysLT receptor antagonists was determined as previously described (Nakagawa et al., 1992), with modifications. Briefly, animals were anaesthetized with urethane (17.5 mmol/kg, i.p.), the body temperature was controlled electronically (Crison 639 K) and maintained at 34.5 °C. The animals were ventilated mechanically through a tracheal cannula, connected to a ventilation pump (Basile 7025) adjusted at a rate of 60 strokes/min and treated with D-tubocurarine (3.9 μmol/kg, i.v.) to prevent spontaneous respiratory movements. For intravenous (i.v.) treatments, compounds were administered by means of a polyethylene catheter inserted into the left jugular vein, and for oral (p.o.) treatments, drugs were given by esophagic gavage. Changes in insufflation pressure were monitored. The basal value of insufflation pressure remained stable for at least 2 h and no significant changes were produced by i.v. saline administration. At the end of the stabilization period (30 min) the guinea-pigs were challenged with i.v. leukotriene D_4 (0.4 nmol/kg). Preliminary experiments showed that at least two reproducible responses to the spasmogen could be evoked at an interval of 60 min in the same animal. To assess the pharmacological modulation of these bronchoconstriction responses by the antagonists under study, compounds were administered before the second challenge with the spasmogen (5 min and 1 h for i.v. and p.o. treatments, respectively). Indomethacin (8.4 μmol/kg, i.v., 10 min before the first challenge with leukotriene D_4) was administered to avoid the contribution of thromboxane A_2 to leukotriene D_4 induced bronchoconstriction.

2.2.3. Leukotriene D_4-induced airway microvascular leakage into guinea-pig trachea

Anaesthetized guinea-pigs were artificially ventilated and a catheter was inserted into the right jugular vein for i.v. administration 1 min after the i.v. administration of the Evans blue (31 μmol/kg, corresponding to 30 mg/kg), the animal was challenged with leukotriene D_4 (0.4 nmol/kg, i.v.) and 5 min later the chest was opened, and Evans blue dye was washed out by perfusion via thoracic aorta with saline (0.9% NaCl, 50 ml/2 min). Trachea was then removed and the dye extracted. Microvascular leakage was evaluated from the amount of Evans blue determined spectrophotometrically at 620 nm wavelenght. Microvascular leakage (ng Evans blue/mg tissue) in treated animals and vehicle treated controls was evaluated by difference with baseline values determined by the i.v. administration of saline and dye.

2.3. Data estimations and analysis

All values are expressed as mean ± standard error of the mean (S.E.M.). Values of $P < 0.05$ were considered significant.

Data from drug competition experiments were analyzed by a non-linear least-squares regression analysis using Equilibrium Binding Data Analysis (EBDA) or LIGAND software (McPherson, Elservier-BIOSOFT, 1983). K_i in experiments of [^{3}H]leukotriene D_4 binding in dU937 cell membranes was estimated by the Cheng–Prusoff equation.

Concentration–response curves of leukotriene D_4-induced $[Ca^{2+}]_i$ mobilization were analyzed by fitting the data with a four parameter logistic equation using the program GraphPad Prism (San Diego, CA) for Macintosh in order to determine EC_{50}. Antagonist affinity was expressed as pK_B (negative logarithm of the antagonist dissociation constant) when "Schild plot" analysis (Arunlakshana and Schild, 1959) showed no significant departure from unity slope. In this case, values were estimated according to the equation: $pK_B = \log_{10}$ [dose-ratio − 1] − $\log_{10}$ [antagonist concentration] (Kenakin, 1993; Jenkinson, 1991). When the antagonism was noncompetitive and/or

pseudoirreversible, the pK_B was calculated by the double-reciprocal plot as described by Kenakin (1993). In practice, a double-reciprocal plot of equieffective concentrations of agonist (A) in the absence (1/A) and in the presence (1/A′) of the antagonist (B) was contructed, and K_B was derived from the equation: K_B=[B]/(slope − 1).

In in vivo studies, EC_{50} values were estimated by non-linear regression analysis using the Hill equation as implemented in GraphPad Software Prism, and compared by means of one-way analysis of variance, whereas time-course experiments were analyzed by means of two-way analysis of variance followed by Bonferroni test for multiple comparisons.

2.4. Materials

MEN91507 (8-[2-(*E*)-[4-[4-(4-fluorophenyl)butyloxy]-phenyl]vinyl]-4-oxo-2-(5-1*H*-tetrazolyl)-4*H*-1-benzopyran sodium salt)), Montelukast, Pranlukast and Zafirlukast were synthesized in Laboratorios Menarini (Badalona, Spain). [^{3}H]leukotriene D_4, [^{3}H]leukotriene E_4, [^{3}H]leukotriene C_4 and [^{3}H]leukotriene B_4 (specific activity 100–240 Ci/mmol) were from NEN Life Science Products (Boston, USA). Cold leukotrienes, the Ca^{2+} ionophore ionomycin, and the Ca^{2+} probe Fura-2-AM were from Calbiochem (La Jolla, CA, USA). All other materials were obtainad from Sigma (St. Louis, MO, USA). For in vitro experiments, all drugs were dissolved daily in distilled water (MEN91507) or DMSO (maximal final concentration 0.02%). For in vivo experiments all compounds were dissolved in DMSO at a concentration of 10^{-2} M and then diluted as indicated.

3. Results

3.1. In vitro studies

3.1.1. Radioligand binding studies in guinea-pig lung and DMSO-differentiated U937 cell membrane preparations

Saturation binding experiments were performed for all the [^{3}H]leukotrienes tested to guinea-pig lung membrane and the following K_d and B_{max} values were calculated: 0.15 ± 0.01 nM and 231 ± 36 fmol/mg proteins for [^{3}H]leukotriene D_4; 0.57 ± 0.01 nM and 159 ± 14 fmol/mg proteins for [^{3}H]leukotriene E_4; 112 ± 18 nM and 93.1 ± 6.3 fmol/mg proteins for [^{3}H]leukotriene C_4 and 0.49 ± 0.22 nM and 203 ± 58 fmol/mg proteins for [^{3}H]leukotriene B_4.

In competition studies, MEN91507 inhibited [^{3}H]leukotriene D_4 and [^{3}H]leukotriene E_4 binding to guinea-pig lung membranes with a K_i values in the subnanomolar range (0.50 ± 0.16 and 0.10 ± 0.06 nM, respectively, Table 1), and no inhibitory effect was observed against [^{3}H]leukotriene C_4 or against [^{3}H]leukotriene B_4 binding at a concentration as high as 10 μM. Similar results were obtained with the $CysLT_1$ receptor antagonist Montelukast, Pranlukast, and Zafirlukast (Table 1).

To evaluate whether MEN91507 blocks $CysLT_1$ human receptor binding, competition studies were also conducted in dU937 cell membranes, which express human $CysLT_1$ receptors after exposure to DMSO. Preliminary saturation binding experiments were performed with both [^{3}H]leukotriene D_4 and [^{3}H]leukotriene C_4 (0.05–10 nM). Analysis by Scatchard plot showed that [^{3}H]leukotriene D_4-specific binding approximates to a one site binding model with a K_d of 0.39 ± 0.14 nM and a B_{max} of 98 ± 64 fmol/mg protein. Even the Scatchard transformation of the specific binding of [^{3}H]leukotriene C_4 was consistent with a single binding site and revealed a K_d value of 10.1 ± 0.8 nM with a B_{max} of 694 ± 77 fmol/mg protein. For both ligands the Hill plot analysis gave a straight line with a coefficient close to unity.

In competition studies MEN91507 inhibited the binding of [^{3}H]leukotriene D_4 to dU937 cell membranes with a K_i value of 0.65 ± 0.29 nM (Table 1). Similar affinity values were obtained with Montelukast, Zafirlukast, or Pranlukast while all these compounds were not able to compete for the binding of [^{3}H]leukotriene C_4 (Table 1). To analyse the nature of the interaction of MEN91507 with the $CysLT_1$ receptors, Scatchard analysis for [^{3}H]leukotriene D_4 binding in dU937 cell membranes were performed in the presence of 1 and 10 nM concentrations of MEN91507. This compound concentration-dependently increased the apparent K_d values

Table 1
Inhibition of [^{3}H]leukotriene specific binding to membrane preparations by MEN91507 and Montelukast

Tracer	MEN91507 K_i (nM)	Montelukast K_i (nM)	Zafirlukast K_i (nM)	Pranlukast K_i (nM)
Guinea-pig lung membranes				
[^{3}H]leukotriene D_4	0.50 ± 0.16	1.30 ± 0.32	0.50 ± 0.03	0.29 ± 0.05
[^{3}H]leukotriene E_4	0.10 ± 0.06	0.12 ± 0.02	2.50 ± 0.33	0.70 ± 0.15
[^{3}H]leukotriene C_4	>10,000	>10,000	>10,000	>10,000
[^{3}H]leukotriene B_4	>10,000	>10,000	>1000	>1000
dU937 cell membranes				
[^{3}H]leukotriene D_4	0.65 ± 0.29	0.60 ± 0.17	0.73 ± 0.34	0.64 ± 0.23
[^{3}H]leukotriene C_4	>1000	>1000	>1000	>1000

Data are expressed as K_i (equilibrium inhibition constant). Each value represents the mean ± S.E.M. of at least four separate competition experiments performed in duplicate.

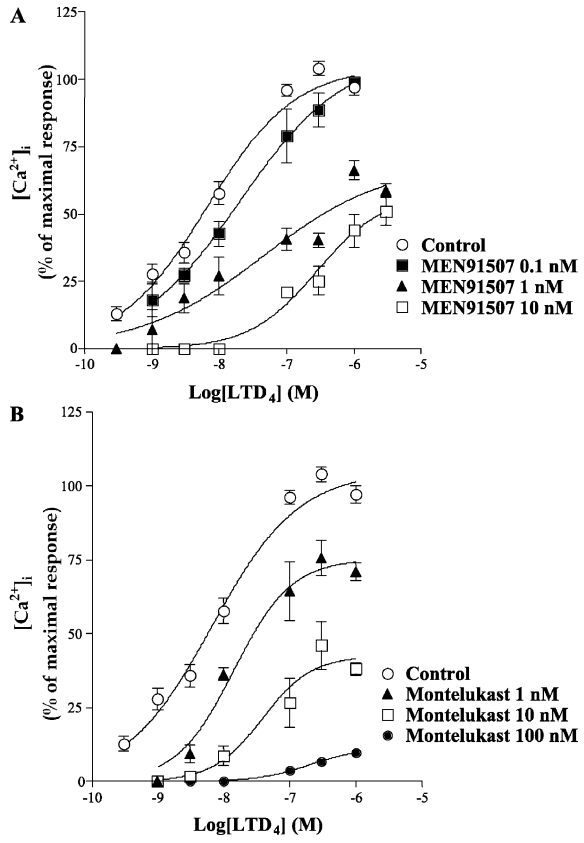

Fig. 2. Antagonism of MEN91507 (A) and Montelukast (B) on leukotriene D_4-induced $[Ca^{2+}]_i$ mobilization in dU937 cells, expressed as the percent of maximal response achieved with leukotriene D_4 in absence of antagonists. Each point is the mean ± S.E.M. of at least four independent experiments.

of [^{3}H]leukotriene D_4 without significantly affecting the B_{max} values (1.46 ± 0.07 nM and 63 ± 4 fmol/mg protein or 84.6 ± 21.7 nM and 74 ± 49 fmol/mg protein in the presence of 1 or 10 nM MEN91507, respectively) suggesting the occurrence of competitive antagonism.

3.1.2. Receptor selectivity and ion channel-binding affinity

The affinity of MEN91507 was determined for many receptors and for the L-type Ca^{2+} channel by using conventional radioligand-binding techniques. MEN91507 showed no relevant binding affinity ($pEC_{50} < 6$) to all the receptors studied (see Section 2.1.3). These results indicate that MEN91507 is a specific and selective antagonist for the $CysLT_1$ receptor.

3.1.3. Leukotriene D_4-induced $[Ca^{2+}]_i$ mobilization in DMSO-differentiated U937 cells

The stimulation of dU937 cells with leukotriene D_4 yielded a rapid increase in $[Ca^{2+}]_i$ that peaked within 10–12 s and then declined more slowly. The maximal response was obtained at 0.1–0.3 μM leukotriene D_4 corresponding to a net $[Ca^{2+}]_i$ increase of 700–800 nM. The analysis of concentration-related curves for leukotriene D_4-induced increase in $[Ca^{2+}]_i$, revealed a pEC_{50} value of 8.2 (95% confidence intervals 7.8–8.6; $n = 11$).

MEN91507 produced a concentration-dependent non-parallel rightward shift of the curve to leukotriene D_4 while depressing maximal response at 1 and 10 nM (Fig. 2A). The apparent pK_B value calculated from the double reciprocal plot method was 10.25 ± 0.15 (95% c.l. 10–10.4; $n = 5$). Similar to that observed with MEN91507, even Montelukast produced concentration-dependent insurmountable antagonism toward leukotriene D_4-induced $[Ca^{2+}]_i$ increase in dU937 cells (Fig. 2B). The depression of the maximal response to the agonist was observed at all the three concentrations tested (Fig. 2B). The apparent pK_B value calculated for Montelukast was 9.42 (95% c.l. 9.31–9.48; $n = 5$). These findings indicated a higher activity of MEN91507 than Montelukast in the inhibition of leukotriene D_4 induced $[Ca^{2+}]_i$ mobilization in dU937 cells. Both compounds behaved as insurmountable antagonists in this assay.

3.2. Antagonist activity of MEN91507 in vivo

3.2.1. In vivo studies on leukotriene D_4-induced bronchoconstriction in guinea-pigs

The inhibitory effect of MEN91507 on leukotriene D_4 (0.4 nmol/kg)-induced bronchoconstriction in anaesthetised guinea-pigs was evaluated and compared with those produced by Montelukast, Pranlukast, and Zafirlukast. The ability of these compounds to inhibit leukotriene D_4-induced bronchoconstriction was assessed after intravenous and oral administration. After intravenous treatment, MEN91507 was significantly ($P < 0.05$) more potent than Montelukast, Pranlukast, and Zafirlukast (Table 2 and Fig. 3A). When compounds were orally administered, MEN91507 completely antagonized the leukotriene D_4-induced bronchoconstriction at doses close to 0.6 μmol/kg (Fig. 3B). The ED_{50} value obtained with MEN91507 was not significantly different from that of Montelukast; even following oral administration, the less potent compounds were found to be Pranlukast and Zafirlukast (Table 2 and Fig. 3B).

The duration of the antibronchoconstrictor activity of oral MEN91507 was studied at 0.6 and 6 μmol/kg. At both

Table 2
Potency of $CysLT_1$ receptor antagonists on leukotriene D_4-induced bronchoconstriction in anesthetized guinea-pigs

Compound	ED_{50} (nmol/kg)	
	i.v. administration	p.o. administration
MEN91507	3.0 ± 0.3	140 ± 90
Montelukast	7.5 ± 1.0[a]	69 ± 6
Zafirlukast	61 ± 6[b]	1540 ± 260[b]
Pranlukast	31 ± 1[b]	1130 ± 70[b]

Compounds were administered 5 min (i.v.) or 1 h (p.o.) before the second challenge with i.v. leukotriene D_4. ED_{50s} (mmol/kg) were calculated by non-linear regression analysis from dose–response plot (Fig. 3a and b). Each value represents the mean ± S.E.M. of 6–11 experiments.

[a] $P < 0.05$ vs. MEN91507.

[b] $P < 0.01$ vs. MEN91507.

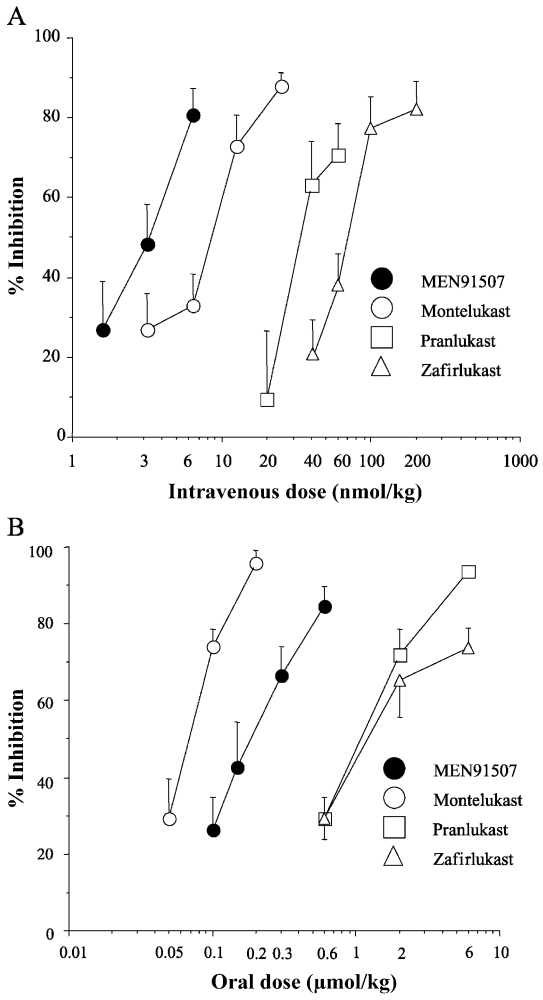

Fig. 3. (A) Intravenous and (B) oral dose–response effect on leukotriene D_4-induced bronchoconstriction in anaesthetized guinea-pig of $CysLT_1$ receptor antagonists. Compounds were administered 5 min (i.v.) or 1 h (o.s.) before second leukotriene D_4 challenge. Curves were fitted by non-linear regression analysis. Data are expressed as % inhibition of bronchoconstriction induced by leukotriene D_4 in the absence of antagonists. Each value is the mean ± S.E.M. of 6–11 animals.

dose levels, MEN91507 inhibited leukotriene D_4-induced bronchoconstriction by >70% up to 8 h after oral administration (Table 3). However, in this case no clear dose-dependency was observed, since 0.6 and 6 μmol/kg lead to

Table 3
Oral effect of $CysLT_1$ receptor antagonists on leukotriene D_4-induced bronchoconstriction in anaesthetized guinea-pigs

	% Inhibition at indicated time post-administration			
Time (h)	1		8	
Dose (μmol/kg)	0.6	6	0.6	6
MEN91507	85 ± 5	98 ± 1	74 ± 10	87 ± 4
Montelukast	90 ± 4	n.t.	92 ± 7	n.t.
Pranlukast	n.t.	100 ± 1	n.t.	90 ± 3

Results are expressed as percent of inhibition (mean ± S.E.M. of 6–11 experiments) of the mean value of bronchoconstriction measured in control animals treated with vehicle alone. N.t.: not tested.

Table 4
Oral effect of $CysLT_1$ receptor antagonists on leukotriene D_4-induced microvascular leakage into guinea-pig trachea, expressed as percent inhibition ± S.E.M. of 6–11 experiments as compared to control group of vehicle treated animals

Compound	% Inhibition at indicated time post-administration			
	Route	Dose (μmol/kg)	1 h	16 h
MEN91507	i.v.	0.4	40 ± 8	n.t.
Montelukast	i.v.	0.4	59 ± 7	n.t.
MEN91507	o.s.	6	84 ± 4	33 ± 8
Montelukast	o.s.	6	87 ± 6	58 ± 8
Zafirlukast	o.s.	6	93 ± 3	47 ± 8
Pranlukast	o.s.	6	87 ± 6	27 ± 2

Compounds were administered 1 h (i.v.) or 1 and 16 h (p.o.) before leukotriene D_4 challenge.

fairly similar inhibitory effect. Comparative experiments were also conducted in the presence of Montelukast (0.6 μmol/kg) and Pranlukast (6 μmol/kg). No significant differences were found among the three $CysLT_1$ receptor antagonists at 1 and 8 h post dose (Table 3).

3.2.2. In vivo studies on leukotriene D_4-induced microvascular leakage in anaesthetised guinea-pigs

MEN91507 (400 nmol/kg, i.v.) inhibited by about 50% the leukotriene D_4 (0.4 nmol/kg)-induced tracheal microvascular leakage when administered 1 h prior to agonist challenge and this inhibitory effect was similar to that caused by Montelukast at the same dose level (Table 4). The effect produced by MEN91507 at 6 μmol/kg was time-dependent and a significant ($P<0.05$) inhibition (33%) was still observed at 16 h post-treatment as compared to vehicle-treated animals (Table 4). These results were similar to those obtained with Pranlukast, Zafirlukast, and Montelukast: indeed a slight but not significant difference was observed in favour of both Zafirlukast and Montelukast at 16-h post-treatment (Table 4).

4. Discussion

MEN91507 is a potent and selective $CysLT_1$ receptor antagonist. Guinea-pig lung membranes have been widely used for the detection and characterization of $CysLT_1$ receptor antagonists: in this assay the high affinity (subnanomolar) binding of [^{3}H[leukotriene D_4 or [^{3}H]leukotriene E_4 was potently displaced (subnanomolar affinity) by MEN91507, Montelukast (Jones et al., 1995) as well as by other well-characterized $CysLT_1$ antagonists (e.g., Aharony et al., 1989). In contrast, neither MEN91507, nor Montelukast (or other selective $CysLT_1$ antagonists) displaced specific [^{3}H]leukotriene C_4 binding in guinea-pig membranes, in agreement with studies showing that [^{3}H]leukotriene D_4 and [^{3}H]leukotriene C_4 binding sites are distinct in terms of distribution, function, and pharmacology (Norman et al., 1987; Carstairs et al., 1988; Jones et al., 1995). A similar

situation is found in DMSO-differentiated human cell line dU937, where [^{3}H[leukotriene D_4 but not [^{3}H]leukotriene C_4 binding was potently displaced (subnanomolar affinity) by both MEN91507, Montelukast, and other $CysLT_1$ receptor antagonists (Frey et al., 1993). In this cell line, most of the [^{3}H]leukotriene C_4 binding has been previously shown to correspond to the microsomal γ-glutathione-*S*-transferase rather than an actual CysLT receptor (Metters et al., 1994), and the lack of effect of MEN91507 and Montelukast on [^{3}H]leukotriene C_4 binding would exclude the interaction of these compounds with γ-glutathione-*S*-transferase. These findings indicated that affinity of MEN91507 vs. [^{3}H[leukotriene D_4 binding at $CysLT_1$ receptors in both guinea-pig and human tissues is similar to that previously reported for Montelukast or other $CysLT_1$ receptor antagonists (Krell et al., 1990; Obata et al., 1992; Jones et al., 1995).

Both MEN91507 and Montelukast antagonized in a concentration-related manner leukotriene D_4-induced Ca^{2+} transients in dU937 cells and the apparent affinity was higher for MEN91507 (pK_B 10.3) as compared to Montelukast (pK_B 9.4). Interestingly, both compounds behaved as insurmountable antagonists in dU937 cells, despite the fact that in binding studies in this system both Montelukast and MEN91507 were competitive ligands. Ca^{2+} transients induced by leukotrienes in dU937 cells are exclusively mediated by the stimulation of $CysLT_1$ receptors, as judged by agonists potency and blockade by selective antagonists (Wetmore et al., 1991). Furthermore, no other CysLT receptor was found in this system (Nothacker et al., 2000). Therefore, the insurmountable antagonism of leukotriene D_4-induced Ca^{2+} transients by both MEN91507 and Montelukast could be putatively attributed to the kinetic of the interactions between leukotriene D_4, $CysLT_1$ receptor antagonists, and their receptors to determine the Ca^{2+} response in dU937 cells. In this contest, it is important to remind that leukotriene D_4-induced response develops in about 10 s, whereas 60 min incubations were performed in binding studies on dU937 cell membranes: we speculate that a long incubation time, as performed in binding experiments may be required to leukotriene D_4 to completely displace the antagonists from $CysLT_1$ receptors, and this may explain the insurmountable antagonism of both MEN91507 and Montelukast observed toward leukotriene D_4-induced Ca^{2+} transients in dU937 cells.

MEN91507, Montelukast, Zafirlukast, and Pranlukast antagonized leukotriene D_4-induced bronchoconstriction in guinea-pigs, following both intravenous and oral administration. When administered intravenously, MEN91507 was the most potent amongst the $CysLT_1$ receptor antagonists tested, since the ED_{50s} for Montelukast, Pranlukast, and Zafirlukast were about 2, 10 and 20 times greater than that of MEN91507. Following oral administration Montelukast was slightly (but non-significantly) more potent of MEN91507, whereas the ED_{50s} of Pranlukast and Zafirlukast were 8 and 11 fold greater than that of MEN91507. The protection afforded by oral administration of MEN91507, Montelukast, and Pranlukast against leukotriene D_4-induced bronchoconstriction was long-lasting, since after 8 h this response was still substantially antagonized. Therefore, although following the oral route of administration the absolute potency of MEN91507 and Montelukast was greater than that of Pranlukast, the duration of the anti-bronchoconstrictor effect of the latter compound was relatively longer than that of both MEN91507 and Montelukast.

In addition to the blockade of airway smooth muscle contractions, $CysLT_1$ receptor antagonists also exerted a potent anti-inflammatory effect at this level. The oral administration of MEN91507, Pranlukast, Montelukast, and Zafirlukast also produced a long-lasting inhibition of leukotriene D_4-induced microvascular leakage. Again, no significant differences were observed in the duration of the anti-inflammatory effect of these antagonists. Differences in the potencies of various antagonists in blocking different effects induced by the same agonist (e.g., leukotriene D_4-induced bronchoconstriction vs. plasma leakage) are not uncommon and could be due to a differential distribution of the drug in the target tissues.

The affinity of MEN91507, Montelukast, Zafirlukast, and Pranlukast, for $CysLT_1$ receptors in guinea-pig lung membranes is comparable, therefore the higher potency of MEN91507 in inhibiting leukotriene D_4-induced bronchoconstriction following the i.v. administration could be indicative of a greater metabolic stability, lower excretion rate, or differential tissue distribution as compared to the other $CysLT_1$ receptor antagonists.

In human tissue (dU937 cells), the affinity of Zafirlukast (K_i = 0.73 nM) and Pranlukast (K_i = 0.64 nM) was comparable to that of MEN91507 (K_i = 0.65 nM) and Montelukast (K_i = 0.60 nM). However, oral doses of 10, 40, and 450 mg/die are respectively needed for Montelukast, Zafirlukast, and Pranlukast to exert a therapeutic effect in asthmatic patients (Markham and Faulds, 1998; Roquet et al., 1997; Tomari et al., 2001). Therefore, although Montelukast was the most potent $CysLT_1$ receptor antagonist after oral administration in both humans and guinea-pigs, the oral effective doses of Pranlukast and Zafirlukast greatly diverge in humans, whereas they are similar in guinea-pigs. Even when considering Montelukast only, its oral bioavalability seems higher in humans than in guinea-pigs (see above), since an oral dose of 10 mg produced a clinical effect that was comparable to an intravenous dose of 7 mg (Dockhorn et al., 2000). All these results indicate the presence of important species-related differences in the pharmacokinetic, metabolic, and absorptive properties amongst different chemical classes of $CysLT_1$ receptor antagonists.

In conclusion, MEN91507 behave as a potent and selective $CysLT_1$ antagonist in guinea-pigs and human in vitro assays. In vivo, MEN91507 shares with the other $CysLT_1$ antagonists anti-bronchospastic and anti-inflammatory effects; since these effects are long-lasting and are also observed following the oral administration, MEN91507 would merit to be tested in humans. If the favourable

pharmacological profile of MEN91507 observed in guinea-pigs also applies for humans, this compound can be proposed for the clinical development in therapy of asthma.

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