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Pharmacological profile of CR3465, a new leukotriene $CysLT_1$ receptor antagonist with broad anti-inflammatory activity

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Abstract

CR3465 (L-Tyrosine, *N*-[(2-quinolinyl)carbonyl]-*O*-(7-fluoro-2-quinolinylmethyl) sodium salt) is a potent antagonist of [^{3}H]leukotriene D_4 ([^{3}H]LTD_4) binding to guinea pig lung preparations, its K_i (4.7±0.7 nM) being comparable with that of montelukast (5.6±0.6 nM). In tracheal strips from standard or ovalbumin-sensitized guinea pigs, CR3465 caused parallel rightward shifts in the concentration–response curves obtained with either LTD_4 or antigen (pA_2, 8.74 and 8.15). Intravenous (i.v.) administration of the agent both antagonized (ED_{50}, 9.9±1.9 μg/kg) and reverted LTD_4-induced bronchoconstriction of anesthetized guinea pigs. CR3465 reduced inflammatory infiltrates in the bronchoalveolar lavage fluid after antigen challenge of sensitized animals, and proved also active in inhibiting phosphodiesterase 3 (PDE3) and phosphodiesterase 4 (PDE4) activities exhibited by human platelets and neutrophils (IC_{50}, 2.01±0.07 and 4.7±0.5 μM). In line with properties shown by phosphodiesterase inhibitors, CR3465 reduced the contractile response of guinea pig airways to histamine and decreased *N*-formyl-Met-Leu-Phe (fMLP)-induced degranulation of human neutrophils (IC_{50}, 13.8 μM). Oral administration (20 mg/kg) of the compound in rats produced a significant (37%) ex vivo inhibition of tumor necrosis factor-alpha (TNF-α) release from lipopolysaccharide-stimulated whole blood. Pharmacokinetic data in the rat demonstrated approximately 100% bioavailability of the agent. We conclude that CR3465 represents a potent leukotriene $CysLT_1$ receptor antagonist with enhanced effects, being also useful for counteracting spasmogenic and inflammatory stimuli other than those elicited by cysteinyl–leukotrienes (Cys-LTs).

Keywords: Leukotriene receptor antagonist; Leukotriene $CysLT_1$ receptor; Cysteinyl–leukotriene; Asthma; Airway inflammation

1. Introduction

Cysteinyl–leukotrienes (Cys-LTs), namely leukotriene C_4, D_4 and E_4, are synthesized de novo by transformation of arachidonic acid via the activity of the 5-lipoxygenase enzyme (Samuelsson, 1983). Experimental research characterized Cys-LTs as potent stimulators of smooth muscle contraction, their effects being of particular relevance in the respiratory system, where they can cause severe bronchoconstriction (Hanna et al., 1981; Griffin et al., 1983). In normal subjects, inhalation of leukotriene D_4 (LTD_4) produces the same degree of airway obstruction as 10,000 times more concentrated inhaled solution of histamine. Moreover, increased synthesis of leukotrienes has been demonstrated in vivo following antigen challenge of allergic subjects (Creticos et al., 1984). Early studies pertaining to Cys-LTs effects have been centered on their contractile activity whereas, more recently, several investigators demonstrated their contribution to airway hyperresponsiveness, mucus plug formation, epithelial cell damage, and airway remodeling (reviewed in Holgate et al., 2003). It is now clear that cysteinyl–leukotrienes are endowed with a variety of proinflammatory actions, and it has been therefore speculated that these mediators might play a pivotal role in the pathogenesis of asthma.

Recent pharmaceutical research in the field of respiratory diseases has sought to make available new agents that specifically and selectively antagonize the actions of

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cysteinyl–leukotrienes. Such an effort has resulted in the development of two classes of drugs, effective in counteracting Cys-LTs pathway, that are now offered for the treatment of asthma: LTD_4 receptor antagonists (montelukast, pranlukast, and zafirlukast) and leukotriene synthesis inhibitors (zileuton; Brooks and Summers, 1996). Cysteinyl–leukotrienes act via two different G-protein-coupled receptors, leukotrienes $CysLT_1$ and leukotriene $CysLT_2$ (Gorenne et al., 1996; Heise et al., 2000). The former appears to be the primary regulator of the deleterious effects that cysteinyl–leukotrienes exert on human airways because selective antagonism of leukotriene $CysLT_1$ receptor, by means of the previously cited compounds, has been shown to improve both asthma symptoms (Suissa et al., 1997) and the underlying inflammatory response (Nakamura et al., 1998). In the clinical setting, leukotriene $CysLT_1$ receptor antagonists were demonstrated to be effective for the treatment of mild to moderate asthma, particularly that associated with inhaled steroid and β-adrenergic agonist therapies (Liu et al., 1996). Moreover, they are highly efficacious in the prevention of exercise-induced bronchoconstriction, and can be used in aspirin-intolerant asthmatic subjects (Manning et al., 1990; Dahlen et al., 1993).

This manuscript illustrates the preclinical pharmacological profile of CR3465 (L-Tyrosine, *N*-[(2-quinolinyl)carbonyl]-*O*-(7-fluoro-2-quinolinylmethyl) sodium salt), a new potent and selective leukotriene Cys-LT_1 receptor antagonist synthesized by Makovec et al. (2001). CR3465 also has additional antiinflammatory activities; altogether, these characteristics are suggestive of a potential use for CR3465 in the treatment of pathologies affecting the respiratory system.

2. Methods

2.1. In vitro studies

2.1.1. Radioligand binding studies

Male Hartley guinea pigs weighing 300–350 g were euthanized by decapitation. Lungs were removed and dissected free from connective tissue. Large airways and blood vessels were cut in small pieces, homogenized and membrane preparation carried out as previously reported (Capra et al., 1998). Protein concentration was determined according to a widely employed method (Bradford, 1976) and crude membrane stored in aliquots at −80 °C until use. Binding experiments were performed after resuspending membranes in cold assay buffer (HEPES-KOH, 10 mM, pH 7.4; $CaCl_2$, 1 mM; glycine, 10 mM; L-cysteine, 10 mM) to a final protein concentration of 1 mg/ml. Reactions, in a final volume of 250 μl, were carried out in polystyrene microplates at 25 °C. In saturation assays, membranes were incubated with increasing concentrations of [^{3}H]leukotriene ([^{3}H]LTD_4) in the absence (control curve) or presence of test substances. Equilibrium binding studies were executed by means of a mixed-type protocol. The first four points of the curve (0.1–0.6 nM) were obtained by using increasing concentrations of labeled ligand (saturation part of the curve), whereas the last four (1–10 nM) were acquired by adding increasing concentrations of unlabeled homologous ligand to a fixed amount of [^{3}H]LTD_4 (homologous competition part of the curve).

In association experiments, membranes were incubated with 0.3 nM [^{3}H]LTD_4 for 45 min in the presence or absence of CR3465. In dissociation assays, membranes were incubated with 0.3 nM [^{3}H]LTD_4 for 30 min to reach equilibrium conditions; then, unlabeled 1 μM LTD_4 (control curve) or 10 μM CR3465 (antagonist curve) were added at definite times (1 up to 90 min). Bound radioligand was separated by rapid filtration, washed twice, and specific binding determined as the difference between the amount of radiolabeled leukotriene bound in the absence or presence of 1 μM LTD_4, representing about 90% of the total binding.

2.1.2. Organ bath studies

Tracheal strips from male Hartley guinea pigs were prepared by cutting through the bands of cartilage. Strips were transferred to a Petri dish containing Krebs–Henseleit solution of the following composition (mM): NaCl, 118.9; KCl, 4.69; $CaCl_2 \times 2H_2O$, 3.33; KH_2PO_4, 1.18; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 25; glucose, 11.1. Strips were then set up at a resting tension of 2 g in 5-ml organ baths containing Krebs–Henseleit solution, maintained at 37 °C and bubbled with a 95% O_2–5% CO_2 gas mixture. Tracheal strips were initially washed with fresh buffer every 15 min, over a 60-min equilibration period. Isometric contractions were monitored using a pen recorder (mod. 7070, Basile, Comerio, Italy). At the beginning of the experiment, acetylcholine (Ach) 300 μM was administered to check the sensitivity of the preparations as well as to determine their maximal contractile response. After further washing and a 15-min equilibration period, test substances or their respective vehicles were added. About 30 min later, cumulative concentration–response curves were obtained with each agonist (LTD_4, 0.1–300 nM; ovalbumin, 0.1–10,000 ng/ml; histamine, 0.003–1000 μM). Only one curve was obtained from each tracheal strip, and the contractile response was expressed as percentage (%) of the maximal contraction achieved with acetylcholine. In experiments performed in the presence of LTD_4, L-cysteine (3 mM) was added to the buffer in order to prevent extensive metabolism of the agonist. In experiments evaluating the potential antagonism of antigen-induced contractions, guinea pigs were sensitized to ovalbumin as described in the in vivo section. Moreover, to make the contractile response specifically leukotriene dependent, isolated tissues were preincubated in the presence of mepyramine (0.3 μM) and indomethacin (10 μM).

2.1.3. Phosphodiesterase 3 (PDE3) and phosphodiesterase 4 (PDE4) activity assay

Purification of human neutrophils (PDE4 activity evaluation) and of S105 fraction from human platelets (PDE3 activity evaluation) was carried out, starting from fresh human blood samples, as described elsewhere (Barnette et al., 1998; Hidaka and Asano, 1976). PDE3 and PDE4 enzymatic activities were assayed by using the Phosphodiesterase [^{3}H]cyclic adenosine 5′ -monophosphate ([^{3}H]cAMP) Scintillation Proximity Assay kit (Amersham Biosciences, Piscataway, NY, USA) according to the manufacturer's instructions. The reaction is based on the breakdown of [^{3}H]cAMP by phosphodiesterase to the corresponding 5′ -monophosphate, which as a linear nucleotide preferentially binds to yttrium silicate beads as compared with cyclic nucleotides. The percentage of basal enzymatic activity (percentage vs. control) occurring in the presence of different concentrations of the tested substances was calculated. Results are expressed as the $IC_{50}\pm$S.E.M. of the agents under investigation.

2.1.4. fMLP-induced degranulation of myeloperoxidase (MPO) from human neutrophils

Human neutrophils were purified from fresh blood. Purity of neutrophils was >97%, as determined by differential cell counts from smears treated with May–Grunwald–Giemsa stain. Cell number was adjusted to $0.75–1.5\times10^6$ cells/ml, depending on the individual donor, in Earle's balanced salt solution, pH 7.4, containing 20 mM HEPES, and 0.1% w/v bovine serum albumin, in the presence of 5 μg/ml cytochalasin B. Cells were incubated 5 min at 37 °C in a shaking water bath and pretreated for additional 5 min with different concentrations of degranulation inhibitors, CR3465 or vehicles, before the addition of 30 nM *N*-formyl-Met-Leu-Phe tripeptide (fMLP). Degranulation reaction was carried out for 30 min and terminated by placing the samples on ice. All samples were centrifuged at $10{,}000\times g$ for 5 min, the supernatant collected and kept frozen until use. MPO activity was assessed by a colorimetric reaction as previously reported (Barnette et al., 1998). Basal and total MPO activities were evaluated in non-stimulated and in sonicated cells, respectively. Results are expressed as percent inhibition of fMLP-induced degranulation.

2.2. In vivo studies

2.2.1. Animals

Male Hartley guinea pigs (Charles River, Calco, Italy) and Sprague–Dawley rats (Harlan, San Pietro al Natisone, Italy) were used for the in vivo and ex vivo experiments. Animals were housed in a temperature- and humidity-controlled room (12-h light/dark cycle) with free access to food and water. All animals used in the studies were cared for in accordance with the principles and guidelines of the Local Government and the regulations of the European Community. Authorization for experimental procedures was granted by the Italian Ministry of Health.

2.2.2. Measurements of bronchoconstriction in anesthetized guinea pig

Anti-bronchoconstriction activity of leukotriene $CysLT_1$ receptor antagonists was determined as previously described (Krell et al., 1987), with some minor modifications. Briefly, guinea pigs were anesthetized by intraperitoneal (i.p.) injection of sodium phenobarbitone (100 mg/kg) supplemented with sodium pentobarbitone (50 mg/kg) dissolved in saline. Animals were mechanically ventilated (60 strokes/min) through a tracheal cannula connected with a ventilator pump (model 7025, Basile, Comerio, Italy); intramuscular gallamine (10 mg/kg) was administered to prevent spontaneous respiratory movements. Airflow, transpulmonary pressure and arterial pressure were monitored by means of a PowerLab/400 computer system (ADInstruments, Sydney, Australia), which integrates air flow and blood pressure to obtain tidal volume and heart rate, respectively. Pulmonary resistance (Rp) and dynamic lung compliance (Cdyn) were calculated using the isovolumetric method. Animals were allowed a 15-min stabilization period before any agent or vehicle administration.

2.2.2.1. Antagonism/reversal of LTD_4-induced bronchoconstriction. Because catecholamine and cyclooxygenase products modulate pulmonary responses to leukotrienes, both RS-propranolol (0.5 mg/kg) and indomethacin (10 mg/kg) were injected intravenously (i.v.) 25 and 30 min before LTD_4 administration. In antagonism studies, guinea pigs were challenged with i.v. LTD_4 (0.25 μg/kg) 10 min after vehicle or drugs administration. In studies evaluating the reversal of LTD_4-induced bronchoconstriction, agonist was intravenously administered in a bolus dose (1 μg/kg) producing an approximate 700% increase in Rp and a 95% decrease in Cdyn. Test drugs or vehicles were administered immediately after achieving the maximal changes in pulmonary parameters. The rate of return to baseline of respiratory function was monitored for 30 min. In both types of experiments, Rp and Cdyn values in the presence of LTD_4 alone were regarded as 100% response (bronchoconstriction peak). Results are expressed as percent variation obtained in the presence of test substances.

2.2.2.2. Histamine-induced bronchoconstriction in anesthetized guinea pig. Surgical procedures were as above indicated. About 10 min before histamine stimulation, test substances or vehicles were administered by i.v. injection. Intravenous dose–response curves to histamine (starting from 2 μg/kg up to 16 μg/kg) were then constructed. After each dose, animals were allowed a 10 min period to reestablish a stable baseline. Only one dose–response curve was obtained from each animal. Bronchoconstriction induced by histamine (increase in Rp and decrease in Cdyn) is expressed as percent change from baseline.

2.2.3. Antigen-induced infiltration of inflammatory cells into bronchoalveolar lavage fluid of sensitized guinea pigs

Male Hartley guinea pigs were treated as previously described (Krell et al., 1987), with some modifications. Animals were actively sensitized by single i.p. administration (1 ml/kg) of 0.1 mg ovalbumin in aluminium hydroxide (Alugel) 2%. On days 21 to 25, sensitized guinea pigs were placed in a transparent chamber (27×20×15 cm) connected with an ultrasonic nebulizer (ULTRA-NEB 99-DEVILBISS) and challenged with 0.5% ovalbumin aerosol for 1 min (spray volume 2 ml/min). Drugs under investigation or vehicle (mannitol 5% w/v) were subcutaneously (s.c.) administered 1 h before and 3 and 7 h after the challenge. Twenty-four hours later, guinea pigs were anesthetized with urethane (1.5 g/kg) and bronchoalveolar lavage performed in situ by using a tracheal tube to instill and subsequently withdraw 10 ml of warmed (37 °C) 0.9% NaCl sterile solution (procedure repeated three times). Lavage fluid was centrifuged (200×*g*, 15 min) and pellet resuspended in 0.5 ml of phosphate-buffered saline solution (PBS) without Ca^{2+} and Mg^{2+}. Total cell number was counted and vitality measured by Trypan Blue dye exclusion. Differential cell counts were made from smears stained with May–Grunwald–Giemsa stain. Three hundred cells per smear were counted, and total population of each cell type calculated.

2.3. Ex vivo studies

2.3.1. Tumor necrosis factor-alpha (TNF-α) release from rat whole blood

A method described by Hartman et al. (1993) was adopted with some modifications. Sprague–Dawley rats weighing 250–300 g (Harlan, San Pietro al Natisone, Italy) were used, fasted 18 h before the experiment, and had water ad libitum. Ninety minutes before blood collection, test compounds or vehicles were orally administered. Whole blood aliquots of 500 μl were incubated in a shaking water bath, at 37 °C for 2 h, in the presence of lipopolysaccharide (5 μg/ml). At the end of the incubation period, samples were placed on ice, 10 mM EGTA added and centrifugation performed at 1500×*g* for 10 min (10 °C). Aliquots of plasma samples were collected and TNF-α release measured by enzyme-linked immunosorbent assay (ELISA) procedure (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Results are expressed as percent inhibition of lipopolysaccharide-induced TNF-α release.

2.4. Pharmacokinetic data in the rat

To determine the pharmacokinetic profile of CR3465 in the rat, the compound was either orally administered as water solution (20 mg/5ml/kg) to Sprague–Dawley rats weighing 250–300 g or injected through the tail vein at a dose of 5 mg/kg. After the administration, blood samples were taken at selected times and plasma CR3465 levels determined by high-pressure liquid chromatography (HPLC), followed by area under curve calculation (AUC).

2.5. Data analysis

Binding curves were analyzed using the nonlinear curve fitting program RADLIG (McPherson, Elsevier-BIOSOFT, 1983), and K_i values estimated by the Cheng–Prusoff equation.

In experiments evaluating the contractile response of isolated tracheal strips, curve analysis was performed by using the Allfit program, which calculates lower and upper plateau, slope and agonist EC_{50} (or the negative logarithm pD_2), and allows the comparison of two or more curves. Antagonist potency was evaluated by the estimation of pA_2 and pK_B values.

IC_{50} and ED_{50} values were determined by linear regression analysis using a statistical software developed by Tallarida and Murray.

Unless otherwise indicated, values are expressed as mean±S.E.M., and Student's *t*-test or one-way analysis of variance (ANOVA) was used for statistical analysis. Values of $P<0.05$ were considered as statistically significant.

2.6. Materials

CR3465 and montelukast were synthesized at Rotta Research Laboratorium (Monza, Italy). [^{3}H]LTD_4 (specific activity, 110 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, USA). Cold LTD_4 and LTC_4 were from Cayman Chemical (Ann Arbor, MI, USA). All other products, unless otherwise specified, were from Sigma (St. Louis, MO, USA).

3. Results

3.1. Basic pharmacological profile of CR3465

3.1.1. Binding studies

In our experimental model, the binding of [^{3}H]LTD_4 was specific, saturable and consistent with a single binding site. The equilibrium parameters for the endogenous ligand, measured in terms of affinity (K_d) and maximal binding capacity (B_{max}), were determined to be 1.16±0.2 nM and 0.665±0.092 pmol/mg protein, respectively. CR3465 concentration-dependently inhibited [^{3}H]LTD_4 specific binding, with a K_i value in the nanomolar range (4.7±0.7 nM). Similar results were obtained with the reference compound montelukast (K_i=5.6±0.6 nM). In competition experimental conditions, the presence of 10 nM CR3465 significantly increased the apparent K_d value of [^{3}H]LTD_4 (4.2±0.9 nM, P=0.007) without significantly affecting B_{max} (0.453±0.084 pmol/mg protein); these results suggest the occurrence of competitive antagonism. Preincubation with CR3465 (10 nM) slowed [^{3}H]LTD_4 binding to its receptor,

causing an apparent fourfold decrease of the association rate, calculated as ratio of the second-order association constants (K_{on}; Fig. 1A). On the other hand, a 10,000-fold excess of CR3465 did not significantly modify prebound [^{3}H]LTD$_4$ dissociation profile (Fig. 1)B, indicating that the antagonistic activity of the compound is principally linked to the association of the endogenous agonist with its receptor. Moreover, the affinity of CR3465 for a variety of other receptor types was evaluated. These experiments showed that the compound had no relevant binding affinity (pEC$_{50}$<6) for any of the following receptors: serotonin and uptake sites, adenosine, opioid and opioid-like, angiotensin-II and substance P. Taken together, these results indicate that CR3465 selectively interacts with the leukotriene CysLT$_1$ receptor subtype.

3.1.2. Organ bath studies

To assess with a functional paradigm the activity shown by the agent in binding assays, we evaluated the effects of CR3465 on LTD$_4$-induced contraction of isolated guinea pig tracheal strips. In this experimental model, LTD$_4$ (0.01–300 nM) generates a concentration-dependent contraction with an EC$_{50}$ in the nanomolar range. Tissue preincubation with increasing amounts of CR3465 produced progressive parallel rightward shifts of the curve obtained in the presence of the endogenous agonist (Fig. 2A). Shild plot analysis yielded a derived pA_2 value of 8.74, and pK_B values were not influenced by CR3465 concentration (8.23±0.35), again indicating the occurrence of competitive antagonism. In contrast, the compound was completely inactive versus contraction to LTC$_4$ obtained in the presence of the gamma-glutamyltranspeptidase inhibitor, L-serine-borate (data not shown). Montelukast caused rightward but not parallel shifts of the LTD$_4$ curve, and lowered the upper plateau in a concentration-dependent way (Fig. 2B). Similar results were found when tracheal strips from ovalbumin-sensitized guinea pigs were stimulated with the antigen in the presence of either CR3465 or montelukast (Fig. 2C and D, respectively). CR3465 inhibited antigen-induced contractions in a concentration-dependent manner, with a pA_2 value estimated to be 8.15.

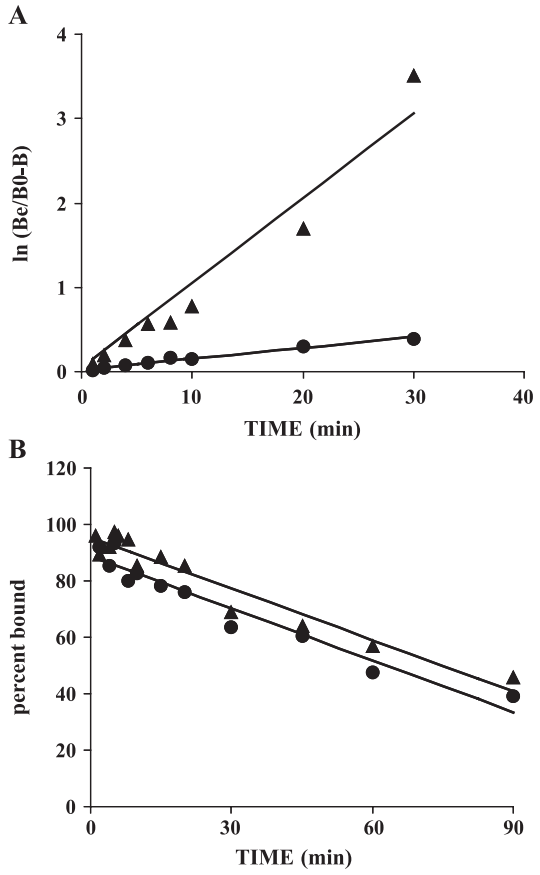

Fig. 1. Kinetic analysis of [^{3}H]LTD$_4$ binding to guinea pig lung membranes. (A) Pseudo first-order plots of the association curve of [^{3}H]LTD$_4$ with its receptor in the absence (▲) or presence of 10 nM CR3465 (●). (B) First-order dissociation plot of [^{3}H]LTD$_4$ receptor complex. Samples were incubated for 30 min and the dissociation reaction initiated by adding either 1 μM LTD$_4$ (▲) or 10 μM CR3465 (●). Data are from two different experiments exhibiting similar results.

3.1.3. In vivo studies

The inhibitory effect of i.v. administered CR3465 on LTD$_4$ (0.25 μg/kg)-induced bronchoconstriction of anesthetized guinea pigs was evaluated and compared with that of montelukast. Intravenous administration of LTD$_4$ provokes considerable increase in pulmonary resistance (Rp) as well as substantial decrease in dynamic lung compliance (Cdyn), reflecting profound bronchoconstriction. Both leukotriene CysLT$_1$ receptor antagonists, when administered prior to leukotriene stimulation, inhibited pulmonary responses in a dose-dependent manner. Montelukast was slightly but not significantly more potent than CR3465, and the ED$_{50}$ calculated for Rp parameter were 3.6±0.8 and 9.9±1.9 μg/kg, respectively. On the other hand, when the antagonists were administered immediately after achieving the maximal change in pulmonary parameters, CR3465 significantly accelerated the rate of Rp and Cdyn return to baseline. A comparison between the effects of CR3465 and montelukast, both tested at 10 μg/kg (close to the above reported ED$_{50}$ values) proved the former as statistically more effective in the reversal of LTD$_4$-induced bronchoconstriction (Fig. 3).

3.2. Antiinflammatory and antispasmogenic activities

We next sought to determine what effects CR3465 might exert in various in vivo and in vitro models relevant to airway inflammation and hyperreactivity to endogenous or exogenous stimuli.

3.2.1. Effects of CR3465 on inflammatory cell infiltration induced by ovalbumin challenge of actively sensitized guinea pigs

In the negative control group, the bronchoalveolar lavage total leukocyte count was 10.2×10^6, consisting almost

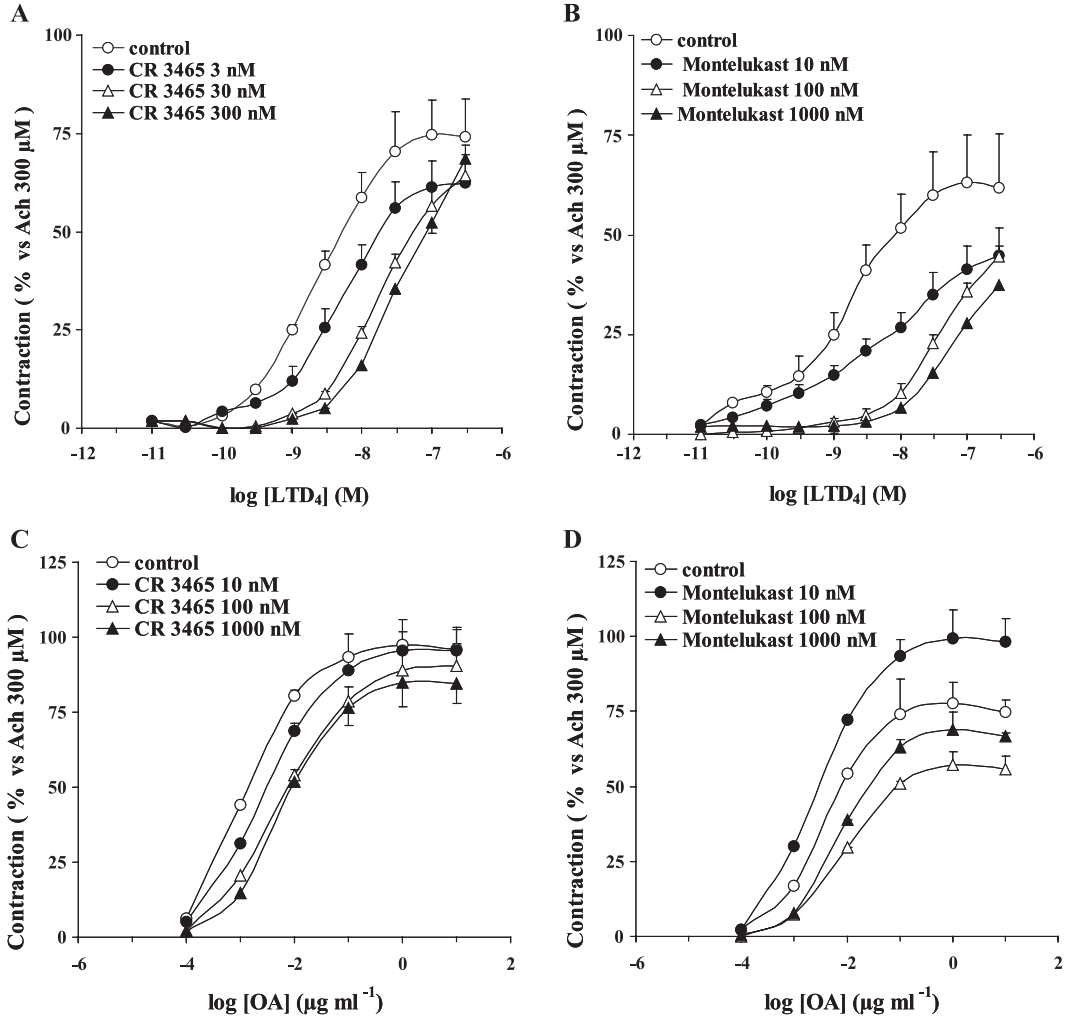

Fig. 2. Effect of CR3465 (A) and montelukast (B) on LTD_4-induced contraction of isolated guinea pig tracheal strips. Each point represents the mean of four replicates; vertical bars show the S.E.M. Effect of CR3465 (C) and montelukast (D) on ovalbumin-induced contraction of isolated guinea pig tracheal strips. Each point is the mean of 3–6 replicates. Data are expressed as the percent of maximal response achieved with acetylcholine.

entirely of alveolar macrophages. Twenty-four hours after antigen challenge, total bronchoalveolar lavage leukocyte count increased to 47.7×10^6, with eosinophils representing almost half of total cell population (21.6×10^6 cells). Treatment of sensitized animals with 10 mg/kg CR3465 produced a significant decrease in total cell count as well as in the absolute number of eosinophils (total leukocyte count, 18.7×10^6; eosinophils, 5.1×10^6 [44%]; Table 1). Identical dose of montelukast, although effective in reducing total leukocyte count (27.8×10^6) and eosinophils' absolute number (13.5×10^6), did not produce statistically significant results as compared with vehicle group.

3.2.2. Effects on phosphodiesterase 3 and 4 activity

In routine screening experiments, we observed that CR3465 was able to inhibit PDE4 enzymatic activity exhibited by human neutrophils, with an apparent IC_{50} of 4.7 ± 0.5 μM. CR3465 was about 6-fold less potent than the selective PDE4 inhibitor rolipram (IC_{50}; 0.8 ± 0.3 μM), but about 2- and 25-fold more potent when compared with the nonselective PDEs inhibitors 3-isobutyl-1-methylxanthine (IBMX) and theophylline (IC_{50}; 11.2 ± 0.5 and 122 ± 9.7 μM, respectively). Moreover, similar results were obtained when using S105 fraction of human platelets, displaying a specific PDE3 activity. Again, CR3465 ($IC_{50;}$ 2.01 ± 0.07), although 10-fold less potent than the specific PDE3 inhibitor milrinone (IC_{50}; 0.20 ± 0.009), was 2-fold more potent when compared with IBMX (IC_{50}; 3.75 ± 0.4).

3.2.3. Effect on histamine-induced contraction of guinea pig tracheal strips: synergism with rolipram

In our experimental conditions, increasing concentrations of histamine provoked cumulative contractions of guinea pig tracheal strips, with a PD_2 value of 5.7 ± 0.14. CR3465 (10–100 μM) did not affect this parameter, but produced a slight reduction of the maximal contractile response generated by the agonist; this decrease reached 22% at the highest concentration tested. Considering the previously observed inhibitory activity of CR3465 on phosphodiesterases and the ability of PDEs inhibitors to modulate the

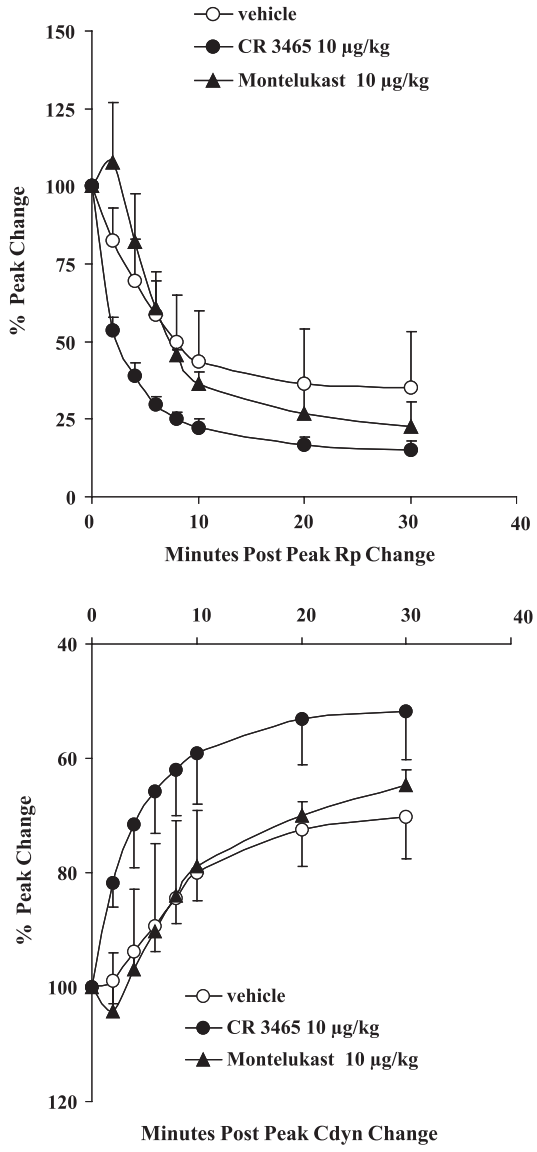

Fig. 3. Reversal of 1 μg/kg LTD_4-induced bronchoconstriction of anesthetized guinea pig. Drugs were i.v. administered after reaching the maximal change in Rp (top) and Cdyn (bottom), and the return rate to baseline was monitored for 30 min. Each point represents the mean±S.E.M. of 2–6 separate experiments. CR3465 (●) treatment produced a statistically significant change of both parameters vs. vehicle-treated (○) and montelukast-treated (▲) animals, as evaluated by all pairwise multiple comparison (Dunn's Method). Rp, pulmonary resistance; Cdyn, dynamic lung compliance.

spasmogenic effect of histamine in the guinea pig trachea (Bernareggi et al., 1999), we used this model to test the compound in combination with the selective PDE4 inhibitor rolipram. As shown in Fig. 4, CR3465 enhanced in a concentration-dependent manner the minor functional anti-histaminergic action exerted by rolipram alone. The synergic effect increased progressively with mounting concentration of histamine (44% inhibition vs. maximal contractile response to histamine), again without affecting its pD_2 value. In comparison, negligible or minor functional antagonism of the contractile response to histamine was observed when tracheal strips were incubated in the presence of montelukast, alone or in combination with rolipram.

Table 1
Evaluation of inflammatory cells infiltration into the bronchoalveolar lavage fluid after challenge of actively sensitized guinea pigs

Treatment (*n*)	Doses (mg/kg)	Total cells ($\times 10^6$)	Eosinophils ($\times 10^6$)
Vehicle (8)	–	47.7±6.6	21.6±3.2
CR3465 (4)	10	18.7±8.5[a]	5.1±2.7[a]
Montelukast (4)	10	27.8±5.8	13.5±3.4

Male guinea pigs were s.c. administered compounds or vehicle (5% w/v mannitol) 3 h before and 3 h and 7 h after the challenge (0.5% w/v aerosol ovalbumin in sterile saline for 1 min). Values are the mean±S.E.M.
[a] $P<0.05$ vs. vehicle group.

3.2.4. Effect on histamine-induced bronchoconstriction in anesthetized guinea pig

Intravenous administration of histamine (dose range: 2–16 μg/kg) in anesthetized animals causes a dose-related

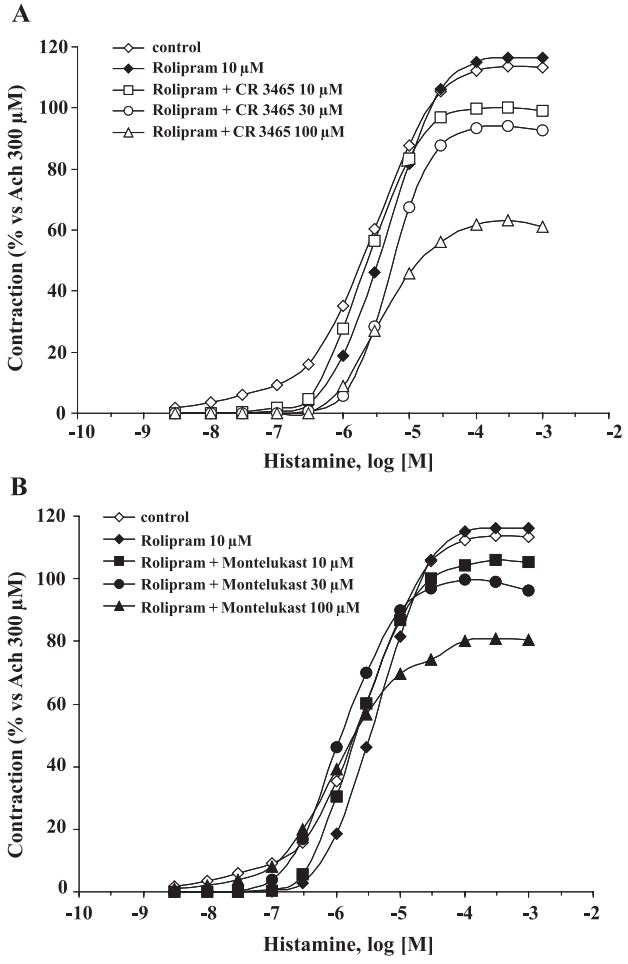

Fig. 4. Effect of CR3465 (A) and montelukast (B), in combination with 10 μM rolipram, on histamine-induced contraction of guinea pig trachea. Data are expressed as the percent of maximal response achieved with acetylcholine. Each point represents the mean±S.E.M. of 2–7 independent experiments.

bronchoconstriction, which can be measured in terms of Rp and Cdyn changes. Consistent with the above described findings, when montelukast or CR3465 were administered intravenously (both at 20 mg/kg) 10 min before challenge, the latter proved more effective in reducing the bronchoconstriction triggered by histamine (Fig. 5). The effect of CR3465 was more pronounced in the Rp parameter, where statistical significance versus vehicle was reached. On the other hand, montelukast was ineffective in this test.

3.2.5. In vitro and ex vivo antiinflammatory activities

Finally, we evaluated the ability of CR3465 to reduce the inflammatory response in widely accepted models such as neutrophil degranulation and whole blood TNF-α release.

N-formyl-Met-Leu-Phe tripeptide (fMLP), a potent chemotactic agent, induces a concentration-dependent degranulation of human neutrophils that can be evaluated through the measurement of MPO enzymatic activity. Basal and fMLP (30 nM) stimulated release of MPO were equal to 2.2±0.3% and 37±3.7% of the total enzyme cellular content, respectively. Rolipram, by increasing cAMP cellular levels, inhibited the fMLP-induced neutrophils degranulation, with a maximal reduction of 52% at 30 μM when compared with fMLP stimulation alone; nonselective PDEs inhibitor IBMX, up to 100 μM, caused a maximal 32% inhibition. As shown in Fig. 6, CR3465 displayed an inhibitory activity comparable with that exhibited by rolipram, with a 75% reduction at 30 μM concentration and an apparent IC_{50} of 13.8 μM.

Tumor necrosis factor (TNF-α) is a cytokine primary synthesized by monocytes and tissue macrophages, and it is thought to play a key role in chronic inflammatory conditions. In vitro stimulation of rat whole blood with lipopolysaccharide (5 μg/ml) resulted in a substantial release of TNF-α (404±56.3 pg/ml). Oral administration of the reference compound dexamethasone (0.3 mg/kg) in rats produced an ex vivo 90% inhibition of this release. When animals were orally treated with CR3465 (20 mg/kg), a statistically significant ex vivo inhibition (37%) of lipopolysaccharide-induced TNF-α release was also observed (Table 2).

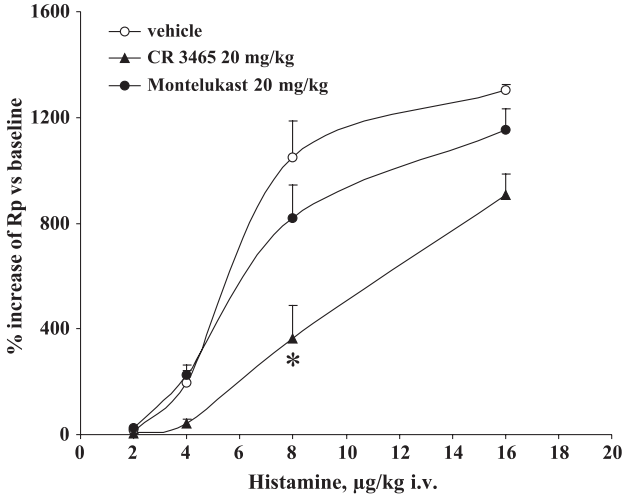

Fig. 5. Effect of CR3465 and montelukast on histamine-induced bronchoconstriction of anesthetized guinea pig. Intravenous dose–response curves to histamine (starting from 2 μg/kg up to 16 μg/kg) were constructed with a 10 min interval between doses. Drugs (both 20 mg/kg: CR 3465 (▲) and montelukast (●)) or vehicle (○) were i.v. administered ten min before the first dose of histamine. Each point represents the mean±S.E.M. of 4–5 separate experiments. *$P<0.05$ vs. vehicle-treated animals.

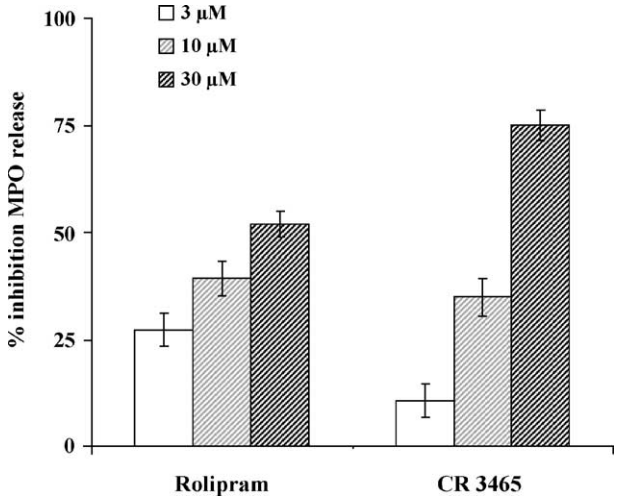

Fig. 6. Effect of CR3465 (IC_{50}, 13.8 μM) and rolipram (IC_{50}, 26.4 μM) on fMLP-induced degranulation of myeloperoxidase from peripheral human neutrophils. Data are expressed as mean±S.E.M. of 4–6 independent experiments for each concentration tested.

3.3. Pharmacokinetics in the rat

In light of the fact that orally administered CR3465 proved active in suppressing inflammatory response ex vivo, we performed a preliminary pharmacokinetic profile of the compound. Oral administration of a 20 mg/kg dose in the rat produced a peak plasma concentration of 20.59 μg/ml between 1 and 2 h after dosing. AUC, evaluated between 0 and 6 h, was determined to be 37.7 μg/h/ml. Intravenous administration of a 5 mg/kg dose produced an AUC, in the same time frame, of 7.38 μg/ml. Ratio between the unit dose AUC (oral) and the unit dose AUC (i.v.) indicated that the oral bioavailability of CR3465 approximates 100%.

Table 2
Evaluation of ex vivo inhibition of TNF-α release from rat whole blood, stimulated with lipopolysaccharide

Treatment (*n*)	TNF-α (pg/ml)	Percent inhibition (%)
Vehicle (10)	404.6±56.0	–
Dexamethasone 0.3 mg/kg (4)	39.3±12.3[a]	90.3
CR3465 20 mg/kg (12)	253.1±34.0[a]	37.4

Rats were orally administered test substances or vehicle 90 min before blood collection. Whole blood aliquots were incubated with lipopolysaccharide (5 μg/ml), at 37 °C for 2 h. Values represent the mean±S.E.M.

[a] $P<0.05$ vs. vehicle-treated group.

4. Discussion

CR3465 is a potent and selective compound designed to specifically antagonize leukotriene $CysLT_1$ receptor. When comparing our results with those reported in previous publications (Krell et al., 1990; Francis et al., 1998), its core pharmacological profile appears to be similar, or even improved, with respect to other chemical entities belonging to the same class. In guinea pig lung parenchymal membranes, CR3465 antagonized [^{3}H]LTD_4 binding with a K_i of 4.7 nM (montelukast, K_i=5.6 nM). The higher montelukast K_i value, as compared with that reported by others (Jones et al., 1994; Cabré et al., 2002), can most likely be ascribed to diverse experimental conditions, as also confirmed by similarly observed differences in terms of [^{3}H]LTD_4 K_d. Nonetheless, because montelukast and CR3465 were evaluated within the same set of experiments, we believe that a direct comparison between the binding affinities of the two compounds is valid and reliable. In isolated guinea pig trachea, CR3465 behaved as an entirely competitive antagonist, causing parallel rightward shifts in the concentration–response curve observed in the presence of LTD_4, and without altering maximal contractile response to the agonist. Conversely montelukast, at the higher concentrations tested (100–1000 nM), significantly lowered the upper plateau of the curve, although this phenomenon was not previously observed by other investigators (Jones et al., 1994). Next, a series of studies in anesthetized guinea pig demonstrated that intravenous administration of CR3465 effectively antagonized/reverted the in vivo LTD_4-induced bronchoconstriction. These findings in guinea pig airways clearly indicate that CR3465 exhibits a leukotriene $CysLT_1$ receptor antagonist activity similar or even greater than that of the reference compound montelukast, both in terms of potency and efficacy. Because potential species-related differences in affinity were reported in binding studies (Aharony, 1998), we understand that these data need to be further confirmed in human tissues.

Such concerns do not apply to the anti-inflammatory actions shown by the agent, given that CR3465 proved effective in various models of inflammation including guinea pig, rat, and human. Airway inflammation, involving a complex network of local mediators, cytokines, and effector cells (Bradley et al., 1991), is believed to play a central role in the pathogenesis as well as in the clinical manifestations of asthma. Thus, a speculative inference suggests that additional antiinflammatory properties shown by a prospective antiasthmatic drug would potentially represent an added value for the management of such a multifactorial condition (Roquet et al., 1997).

In our study, CR3465 administration was associated with a dramatic reduction of total leukocytes and eosinophils in the bronchoalveolar lavage fluid after antigen challenge of actively sensitized guinea pigs, its effect being greater than that of the reference compound montelukast. These results are in agreement with those published in a recent manuscript (Wu et al., 2003), reporting that doses of montelukast higher than 10 mg/kg were necessary to produce a significant decrease of inflammatory cell infiltrates.

Moreover, intravenous administration of CR3465 was able to functionally inhibit histamine-induced bronchoconstriction in vivo. An inhibitory action versus histaminergic stimulus was detected as well in guinea pig isolated tracheal strips, where the compound also exhibited a synergic effect in combination with the PDE4 inhibitor rolipram. These findings are in line with previous studies (Bernareggi et al., 1999) showing that, in histamine-challenged guinea pig tracheal strips, an antispasmogenic effect was only observed with agents exhibiting a mixed PDE3/PDE4 inhibitory action. In experiments performed in the presence of both CR3465 and rolipram, the enhanced inhibition of the contractile response to the agonist can most likely be explained by the fact that CR3465 appears to be a 2.5-fold more potent inhibitor of PDE3, compared with PDE4. Clinical outcomes have recently pointed out that administration of leukotriene $CysLT_1$ receptor antagonists decreases the need for rescue treatment with β-adrenoceptor agonists, and that the additive effects of these two drug classes may be required while treating patients with asthmatic bronchoconstriction (Drazen et al., 1999). Considering the interest in PDE4 as a molecular target for new anti-inflammatory and antiasthmatic pharmacotherapies (Giembycz, 1992), the fact that CR3465 also possesses a phosphodiesterase inhibitory action seems in agreement with this scenario, where cAMP levels in airway smooth muscle are at issue. Consistent with previous reports on antiinflammatory effects shown by PDEs inhibitors (Thorphy and Undem, 1991), CR3465 was effective in reducing fMLP-induced degranulation of human neutrophils; even more importantly, oral administration of the compound proved active in decreasing ex vivo TNF-α release from rat whole blood stimulated with lipopolysaccharide. This latter result denotes high bioavailability after oral dosing in the rat because antiinflammatory actions of CR3465 are exerted at concentrations that exceed by far those needed to function as a cysteinyl–leukotriene antagonist. This is consistent with our pharmacokinetic data, which demonstrated an approximate 100% bioavailability after oral administration of the compound in rats. In this respect, Wu et al. (2003) verified that high-dose montelukast was required to produce antiinflammatory effects in an animal model of acute asthma, mediated through the suppression of T helper type-2 (Th2) cytokines.

Finally, antiinflammatory properties displayed by CR3465 can be positively weighed up from the larger perspective of the clinical employment of leukotriene antagonists. Recent literature primarily describes leukotriene $CysLT_1$ receptor antagonists as antiinflammatory drugs, their use being more preventive rather than rescue therapy for asthma exacerbations (Devillier et al., 1999). Long-term clinical trials have successfully evaluated the effectiveness of leukotriene antagonists in diminishing the

incidence of acute asthmatic outbreaks and the need for glucocorticoid rescue treatment (Spector et al., 1994; Reiss et al., 1996). Nonetheless, a severe condition such as asthma requires a continuous research effort in order to develop new agents allowing better disease management.

In conclusion, CR3465 represents a potent and selective cysteinyl–leukotriene antagonist, additionally endowed with a number of antiinflammatory effects. These characteristics, if confirmed in humans, would make the compound a promising drug for asthma therapy.

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