INVERSE AGONIST ACTIVITY OF SELECTED LIGANDS OF THE CYSTEINYL-LEUKOTRIENE RECEPTOR 1.*

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Abbreviations
BSA, bovine serum albumin; cysLT, cysteinyl leukotriene; DMEM, Dulbecco’s modified eagle medium; FBS, fetal bovine serum; GPCR, G-protein coupled receptor; IP, inositol phosphate; LiCl, Lithium Chloride; PBS, phosphate buffered saline; RT, room temperature; WT, wild type.

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Abstract

Cysteinyl leukotrienes (cysLT) are associated with several inflammatory processes, including asthma. Due to this association, considerable efforts have been invested in the development of antagonists to the CysLT receptors (CysLT₁R). A large number of these molecules have been shown to specifically interact with CysLT₁R but little is known about their impact on the conformation of the receptor and its activity. We were especially interested in possible inverse agonist activity of the antagonists. Using a constitutively active mutant (N106A) of the human CysLT₁R and the wild-type (WT) receptor co-expressed with the Gαq subunit of the trimeric G-protein, we were able to address this issue with ligands commonly used in therapy. We demonstrated that some of these molecules are inverse agonists whereas others act as partial agonists. In cells expressing the CysLT₁R mutant N106A exposed to Montelukast, Zafirlukast or MK571, the basal inositol phosphate production was reduced by 53±6, 44±3 and 54±4 percent, respectively. On the other hand, BayU9773 and LY171883 acted as partial agonists and REV 5901 as a neutral antagonist. However in cells expressing CysLT₁R and Gαq all antagonists used had inverse agonist activity. The decrease in basal inositol phosphate production by ligands with inverse agonist activity could be inhibited by a more neutral antagonist, confirming the specificity of the reaction. We demonstrate here that Montelukast, MK571 and Zafirlukast can act as inverse agonists on the human CysLT₁ receptor.
The cysteinyl leukotrienes, LTC₄, LTD₄ and LTE₄ are lipid mediators generated de novo from membrane-associated arachidonic acid. They are important contributors in the pathophysiology of several inflammatory disorders, in particular of human bronchial asthma (Evans, 2002). Different antagonists such as Montelukast, Pranlukast and Zafirlukast have been used, or suggested, as novel therapeutic agents. Binding and functional studies have provided evidence that the biological effects of the CysLTs are mediated via G-protein coupled receptors. Recently, the cloning and molecular characterization of the CysLT₁ receptor cDNA was reported (Lynch et al., 1999; Sarau et al., 1999). Previous reports on signalling of the CysLT₁ receptor indicated limited signaling through Gαᵢ/o-linked pathways (Lynch et al., 1999), probably via Gαᵢ₃, as previously suggested by Sjölander’s group (Adolfsson et al., 1996).

It has been established that many GPCRs can exist in a spontaneously active form in the absence of agonist (Costa et al., 1992; Lefkowitz et al., 1993; Chidiac et al., 1994). This agonist-independent activity has mostly been observed in cell lines or transgenic mice in which receptors were overexpressed or mutated (Barker et al., 1994; Chidiac et al., 1994; Bond et al., 1995; Newman-Tancredi et al., 1997). Many substitutions resulting in constitutively active conformation are conserved among most GPCRs. Among these, the substitution of an asparagine of the second transmembrane segment to alanine and isoleucine/leucine of the third intracellular loop to an arginine created constitutively active mutations in the PAFR and other receptor (Parent et al., 1996a). In the CysLT₁R, the substitutions of isoleucine 232 of the third intracellular loop to an arginine (I232R) and of asparagine 106 of the second transmembrane segment to alanine (N106A) stabilizes the active form and leads, in COS-7 cells, to a significant increase in the basal production of inositol phosphates (IP). In previous studies, we and others
demonstrated that coexpression of WT receptor and G proteins induced higher inositol phosphate (IP) production, allowing the evaluation of inverse agonist effects (Burstein et al., 1997; Bakker et al., 2001; Dupre et al., 2001).

Constitutively active mutant receptors have been a valuable tool to demonstrate that certain ligands stabilize inactive conformations. Those ligands are known as inverse agonists, given they have the opposite effect of agonists (Milligan et al., 1995). In view of the fact that inverse agonist activity and constitutively active receptors have been shown with over-expressed or mutant receptor proteins, the physiological relevance of the process has been debated. However, recently, several receptors have been shown to be constitutively active in vivo implying that inverse agonists may have preferred therapeutic applications (Adan and Kas, 2003; Schwartz et al., 2003; Seifert and Wenzel-Seifert, 2003).

Until now, various CysLT1R ligands were loosely classified as antagonists, thus as molecules that interfere with the agonist (LTD4) activation of the receptor. In this study, we characterize the effects of these molecules on the activated state of the CysLT1R in order to define which ones have inverse agonist, partial agonist or neutral antagonist (no effect on activation) properties.

By expressing either the combination WT/Gαq or the N106A mutant of CysLT1R, the two different models permitted us to study whether the properties of the ligands would vary depending on the structural conditions leading to the constitutive activation of CysLT1R.
Methods

Materials

Montelukast (1µM) was obtained from Merck-Frosst (Pointe-Claire, Qc, Canada) and Zafirlukast (1µM) from AstraZeneca (Lund, Sweden). BayU9773 (1.5µM), LY171883 (6µM), REV5901 (10µM) and MK571 (1µM) were purchased from BIOMOL (Plymouth Meeting, PA, USA).

Cell culture and transfections

COS-7 cells were grown in DMEM high glucose (Invitrogen, Burlington ON, Canada) supplemented with 10% FBS (Sigma, Oakville ON, Canada) and transfected using FuGENE-6 (Roche, Mississauga ON, Canada). Cells were plated at a density of 2.5x10^5 cells/well in 6-well plates and the following day, transfected exactly as instructed in Roche's protocol, using 2µl of FuGENE-6 and 1µg of DNA. Experiments were carried out 2 days after transfection.

Radioligand binding assay

^3H-LTD₄ (DuPont NEN Life Sciences Products, Boston, MA) binding reactions were performed, as previously described by Sarau et al. (Sarau et al., 1999) with some modifications. Reactions were carried on whole COS-7 cells at pH 7.4 transfected with DNA encoding a cmyc epitope tagged (N-terminus) CysLT1R in pcMV-intron. Cells were harvested, washed twice with PBS and resuspended in Tris-based binding buffer (10 mM CaCl₂, 10 mM MgCl₂, 10 mM glycine, 10 mM cysteine, pH 7.4). The binding assays were done on 5x10⁴ cells in a total volume of 0.25 ml of the same buffer, containing 1 nM ^3H-LTD₄, at RT for 60 min. In some experiments, different antagonists were also included in the mix to compete with the radioligand binding on the
receptor. Binding reactions were stopped by centrifugation, washed with Tris-base buffer and cells were harvested. The cell-associated radioactivity was measured by liquid scintillation.

*Inositol phosphate determination*

COS-7 cells were cotransfected with DNA encoding the $\alpha_q$ subunit of the human trimeric Gq-protein and a cmyc-epitope tagged (N-terminus) WT or a mutant receptor (I232R or N106A). The following day, cells were labelled for 18-24h with 5 $\mu$Ci/ml of $^3$H-myo-inositol (Amersham-Pharmacia Biotech, Piscataway, NJ) in DMEM (high glucose, without inositol) (Invitrogen, Burlington ON, Canada). After labelling, cells were preincubated for 20 min at 37°C in presence of different CysLT1R antagonists. LiCl was then added at a final concentration of 20 mM and cells incubated at 37°C for 55 min. The reaction was terminated after 30 min by the addition of perchloric acid. After an incubation of 30 min on ice, inositol phosphates were extracted and separated on Dowex AG1-X8 columns (Bio-RAD Laboratories, Hercules, CA) as previously described (Parent et al., 1996b). $^3$H-labeled inositol phosphate levels were then evaluated by liquid scintillation.

*Receptor sequestration*

The evaluation of receptor sequestration was done on COS-7 cells transiently expressing a cMyc-epitope tagged (N-terminus) WT receptor. Cells were exposed to medium or to different CysLT1R ligands at 37°C for 30 min in Tris-base buffer. Cells were then harvested, washed with PBS, and incubated at 16°C with or without anti-cMyc antibody (clone 9E10; American Type Culture Collection, Manassas, VA). After 90 min, cells were washed with PBS and incubated for an additional hour with fluorescein isothiocyanate-conjugated goat anti-mouse antibody
(Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Receptor expression on the cell surface was then evaluated using a FACScan flow cytometer (Becton-Dickinson, Oakville ON, Canada).

Statistical analysis

Statistical analyses were done using GraphPad Prism 3.0 software following the methods and equations described in Motulsky and Christopoulos linear and non linear regression guide provided with the software. Student’s t test was used where applicable.
Results

To further amplify the effect of the basal activated state of CysLT1R, COS-7 cells were co-transfected with the cDNA of the human CysLT1R and $G_{\alpha q}$. Other members of the $\alpha_q$ family ($G_{\alpha 11}, G_{\alpha 14}, G_{\alpha 16}$) were tested, but their effect on basal inositol phosphate production was lower than that of $G_{\alpha q}$ (results not illustrated). With the increase in basal activity, we were able to use the WT receptor to test both antagonist and inverse agonist properties of various ligands.

Functional characterization of the mutant receptors

We made two substitution mutants (asparagine 106 of the second transmembrane segment to alanine and isoleucine 232 of the third intracellular loop to arginine) to create constitutively active mutants of CysLT1R. These residues are known in several GPCRs to alter the basal state of the receptors, allowing them to be constitutively activated. The binding characteristics of the WT CysLT1R, the mutants receptors I232R and N106A were examined in transiently transfected COS-7 cells using the agonist LTD4. Figure 1 shows that a small difference in the affinities of the WT and the N106A mutant receptor for $^3$H-LTD4 could be observed. The IC$_{50}$ value for WT CysLT1R was 26 ± 2 nM, whereas it was 16 ± 4 nM for the N106A mutant. The Bmax values for the WT and N106A receptors were of 287 000 ± 29 000 and 283 000 ± 19 000 sites per cell, respectively. The I232R mutant did not bind LTD4, despite similar cell-surface expression of all three receptors, as examined by flow cytometry with an anti-Myc antibody (results not shown). However, the I232R mutant had very high basal activity, as shown by IP production (Table I), producing more than 3 times the level of IPs spontaneously than the WT after ligand stimulation, whereas the basal production of WT/$G_{\alpha q}$ or N106A was close to IP production of the stimulated WT receptor.
Efficiency of CysLT₁R antagonists

Next, we confirmed the antagonist activity of indicated compounds using transfected COS-7 cells. Figure 2A and 2B show competition isotherms for the WT and N106A CysLT₁ receptors, respectively. The $K_d$ and $K_i$ values of LTD₄, MK571, Zafirlukast and Montelukast in competition with $^3$H-LTD₄ are represented in Table II. These compounds are all characterized as CysLT₁R ligands and they effectively interfered with $^3$H-LTD₄ binding to both WT and N106A mutant receptors. Their efficacy in blocking LTD₄-stimulated inositol phosphate production in cells expressing the WT CysLT₁R and $G_{αq}$ was also examined (Figure 3). Concentrations described in literature for their maximal efficacy were used for each experiment. Montelukast (1µM), MK571 (1µM), BayU9773 (1.5µM), Zafirlukast (1µM), LY171883 (6µM) and REV5901 (10µM) were all efficient antagonists, reducing the levels of LTD₄-induced inositol phosphate production by 94±4%, 98±2%, 68±10%, 91±6%, 80±11% and 52±6%, respectively. Similar results were obtained with N106A mutant receptor (data not shown).

Inverse agonist activity of selected CysLT₁ ligands

COS-7 cells expressing WT + $G_{αq}$ or N106A were used for the following experiments. Figure 4A shows that all of the ligands tested had a partial inverse agonist activity on the WT + $G_{αq}$ receptor corresponding to a reduction of approximately 20-25% of basal IP levels (p<0.05). However, when we used the N106A mutant receptor, only Montelukast, MK571 and Zafirlukast were able to reduce the basal level of inositol phosphate production by 28±5% (p<0.03), 27±8% (p<0.03), and 16±6% (p<0.04), respectively (Figure 4B). LY171883 (+38±17%; p<0.05) and
BayU9773 (+20±9%; p<0.05) demonstrated partial agonist activity, whereas REV5901 acted as a neutral antagonist (4±8%; p>0.05).

Extended concentration-response curves for Montelukast, Zafirlukast and MK571, which exhibited efficient inverse agonist activity, were compared in cells coexpressing WT + G_{αq} and in cells expressing the N106A mutant receptor. When the WT/G_{αq} combination was used, the EC_{50} values of Montelukast, MK571 and Zafirlukast were of 1.3nM, 22nM and 32nM, respectively (Figure 5A). However, in cells expressing the mutant receptor N106A, MK571 (EC_{50} : 0.3nM) was active as an inverse agonist (p<0.02) at lower concentrations than either Montelukast (EC_{50} : 11nM) or Zafirlukast (EC_{50} : 1.8nM) (Figure 5B). At high concentrations, the effect of each antagonist was similar.

The action of an inverse agonist should be inhibited by an antagonist, in the same way as a full or partial agonist would be blocked by an antagonist. We treated COS-7 cells overexpressing N106A mutant receptor with REV5901 (10µM), which acted as a neutral antagonist, followed by stimulation with Montelukast (1µM), MK571 (1µM) or Zafirlukast (1µM) (Figure 6). While Montelukast, MK571 and Zafirlukast inhibited basal IP production, pre-treatment with REV5901 effectively antagonized the inverse agonist-induced reduction of inositol phosphate production.

Receptor sequestration

Following ligand binding to the receptor, a cascade of events often leads to sequestration of the receptor from the cell surface. Since lower numbers of cell-surface receptors could produce the same phenomenon of decreased basal activity as inverse agonists, we examined whether
indicated molecules induced an accelerated internalization of the receptor. Receptor expression on the cell surface was assessed using flow cytometry. As shown in Figure 7, only LY171883, which may have partial agonist activity, was able to induce WT CysLT1R internalization at a level (31±5%) comparable to that induced by the agonist LTD4 (35±2%). The other compounds did not induce receptor internalization and actually increased cell surface receptor expression by 25% to 66%. In contrast, the N106A mutant receptor expression was not affected by the antagonists, except by the partial agonist LY171883 which promoted internalization of the receptor (22±7%).
Discussion

It has been shown in the context of several GPCRs that certain molecules could act as inverse agonists and reduce the levels of spontaneous receptor activity and functional cellular responses (Chidiac et al., 1994; Samama et al., 1994; Shryock et al., 1998; Spadoni et al., 1998). Here, we report that molecules known to antagonize diverse responses to LTD$_4$ can also inhibit spontaneous activity of CysLT$_1$R.

A two-state equilibrium model of receptors can illustrate the inverse agonist activity of certain ligands. Many GPCRs can be spontaneously active, suggesting a two-state conformation, an equilibrium between active and inactive conformational states in the absence of agonist (Leff, 1995; Milligan et al., 1995; Leurs et al., 1998). Compounds displaying inverse agonism should have a higher affinity for the inactive state compared to the active conformation, resulting in a decrease in the proportion of receptors in an active conformation and a reduction in the basal activation of effector mechanisms (Samama et al., 1994). It has been proposed for opiate receptors, that the compounds with inverse agonist activity have a preferential affinity for the free receptor over the receptor-G-protein complex and thus suppress agonist-independent activity (Costa et al., 1992). Our results show that although all the different molecules assayed had inverse agonist activity on WT+G$_{\text{q}}$ receptor, only Montelukast, MK571 and Zafirlukast decreased the basal level of IP production in the constitutively active N106A mutant receptor. Interestingly LY171883 and BayU9773 acted as partial agonists whereas REV5901 acted as a bona fide neutral antagonist, indicating that the mutant was probably structurally different from the active form of the WT receptor.
It is interesting that the selected ligands had such diverse effects on the two receptor systems tested, with the mutant receptor being able to effect different responses to the individual antagonists whereas the WT receptor perceived all these ligands as partial inverse agonists. The N106A has a higher spontaneous production of IPs and higher affinity for the agonist than the WT and we may therefore speculate that more receptors are found in the active conformation. However, some of the ligands with inverse agonist activity also have a higher affinity for the mutant that for the WT receptor which does not seem in agreement with the hypothesis that these molecules would prefer the inactive or G-protein uncoupled receptor. The efficacy of inverse agonist activity may depend of the structural properties of every ligand-receptor-G-protein assembly. Alternately, these antagonists could act to stabilize the interaction of receptor-G-protein but would block the G-protein into an inactive form, as was seen for the CB1 cannabinoid receptors and SR141716A (Bouaboula et al., 1997).

In a previous study, we also showed that in COS-7 cells expressing two different constitutively active receptors the effects of various ligands were not identical and varied according to the constitutively active receptor model (Dupre et al., 2001). Similarly, a mutant of the H2 receptor which had a limited response to histamine also failed to respond to an inverse agonist thus differing from WT receptor response (Smit et al., 1996).

We also studied whether the decrease in signaling by inverse agonists could be attributed to an increased sequestration of the receptors from the cell surface. It was shown that inverse agonists could induce the internalization of the CCKA receptor (Roettger et al., 1997), although increased cell surface expression has been reported for other receptors after inverse agonist treatment (Smit et al., 1996; Lee et al., 1997; Zhu et al., 2000). Our results showed that none of the three more
potent inverse agonists (Montelukast, MK571 and Zafirlukast) induced receptor sequestration, rather, they all promoted cell surface expression. Increased receptor expression has usually been seen after prolonged treatment of cells with the inverse agonists and possibly implicated the activation of cellular pathways resulting in resensitization and upregulation of receptor expression (Smit et al., 1996; Lee et al., 1997; Zhu et al., 2000). However, this would not apply under our conditions since our treatment with inverse agonists was relatively short. The increased cell surface expression could be due to the stabilization of the receptor, either intracellularly in order to increase receptor maturation and expression (Petaja-Repo et al., 2002) or by stabilizing the receptor on the cell surface (Wilson and Limbird, 2000; Pauwels and Tardif, 2002). As there is no indication that the antagonists we used in our experiments are cell permeable we would favor the hypothesis that the inverse agonists possibly stabilize constitutively active receptor on the cell surface and possibly interfere with the constitutive internalization process. Other studies proposed that some ligands could stabilize the constitutively activated receptors, but only inverse agonists and not agonists could block internalization and down-regulation (Li et al., 2001). It is possible that the distinct conformations induced by inverse agonists are more resistant to the actions of cellular machinery leading to internalization and down-regulation than those induced by neutral antagonists or agonists (Li et al., 2001). Another group showed that inverse agonists could change the conformation of WT or constitutively activated mutant β2AR receptors to a different one than agonist (Gether et al., 1997). In our case, not all ligands of the receptors could increase cell surface receptor density as CysLT1R sequestration was induced by the agonist LTD₄ and the partial agonist LY171883. Moreover, none of the inverse agonists tested changed the cell-surface expression of the N106A constitutively active mutant receptor. Only the agonist and
partial agonist promoted receptor internalization with this mutant receptor. These results also indicate that the decrease in basal activity induced by selected ligands is not due to an accelerated rate of receptor internalization.

The role of CysLTs in inflammation has been studied intensively and emphasis has been placed on human asthma (Hui and Funk, 2002). Montelukast and Zafirlukast are used as prophylactic and chronic treatment of asthma and can be used as first-line therapy in the case of patients with low responses to corticosteroids (Smith et al., 1993; Diamant et al., 1999; Jarvis and Markham, 2000). Although CysLT₁R antagonists significantly reversed almost all of the morphological aspects of chronic asthma (Henderson et al., 2002), no studies addressed the potential inverse agonist effect of the antagonists on the CysLT₁ receptor and cell signalling. CysLTs have been shown to induce smooth muscle cell proliferation and hypertrophy in conjunction with other known mitogens (Cohen et al., 1995; Amishima et al., 1998; Panettieri et al., 1998). We have also shown recently that upregulation of CysLT₁ by selected cytokines was associated with bronchial smooth muscle cell proliferation in response to LTD₄, blockable by Montelukast (Espinosa et al., 2003). There was no effect on cell surface expression of CysLT₁ in the time frame studied, contrast to the transfected system used in this report. This underlines the importance of the possible cell-specific effects of some molecules. Given the very preliminary indications in the cardiovascular system, that inverse agonists may be of greater benefit in reducing mortality, under specific conditions (Callaerts-Vegh et al., 2003), this may lead us to speculation that ligands of the CysLT₁ with inverse agonist activity may also be of different benefit from those with neutral antagonist, or partial agonist activity.
In this work, we demonstrated that Montelukast, MK571 and Zafirlukast have inverse agonist activity on constitutively active receptors overexpressed in COS-7 cells. These inverse agonists decrease spontaneous inositol phosphate production, without changing the receptor sequestration rate. Our results also demonstrate that the inverse agonist effect of Montelukast, MK571 and Zafirlukast can be inhibited by REV5901, a neutral antagonist. By comparing WT CysLT₁ coexpressed with Gαq and the N106A mutant receptor, we show that the potential of inverse agonist activity can differ from one constitutively active receptor to another. This could possibly be explained by conformational differences and may indicate that a molecule with inverse agonist activity will not necessarily have the same activity on all constitutively active receptors. Future studies of the relationship between ligand structure and their properties may make it possible to understand the fine points of CysLT₁ antagonists which are already used in asthma therapy. It may also lead to the development of new molecules which could induce short or long-term state of unresponsiveness without inducing an effector cascade.
References:


Parent JL, Le Gouill C, Rola-Pleszczynski M and Stankova J (1996b) Mutation of an aspartate at position 63 in the human platelet-activating factor receptor augments binding affinity but...


Footnotes:

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Legends for figures:

Figure 1. Competition binding isotherms of $^{3}$H-LTD$_{4}$ by LTD$_{4}$.

$^{3}$H-LTD$_{4}$ binding was measured as indicated under “Methods” in COS-7 cells transiently expressing the WT CysLT$_{1}$R or N106A mutant receptor. The results are expressed as the means ± s.e. of three independent experiments, each done in duplicate.

Figure 2. Effect of CysLT$_{1}$R antagonists on $^{3}$H-LTD$_{4}$ binding to wild-type CysLT$_{1}$ receptor

Binding reactions were performed on COS-7 cells transiently expressing (A) WT CysLT$_{1}$ or (B) N106A receptor in presence of various concentration of indicated antagonists as described in the Methods. The results are expressed as the means of three independent experiments, each done in duplicate.

Figure 3. Efficiency of indicated antagonists on blocking LTD$_{4}$-induced inositol phosphate production in COS-7 cells overexpressing wild-type CysLT$_{1}$R and G$_{aq}$

Inositol phosphate production was measured after a 20 min pre-treatment with antagonists, followed by stimulation with LTD$_{4}$ (5x10$^{-7}$M). The concentrations of antagonists used were Montelukast (1µM), MK571 (1µM), BayU9773 (1.5µM), Zafirlukast (1µM), LY171883 (6µM) and REV5901 (10µM). The results are expressed as the means ± s.e. of three different independent experiments, each done in triplicate.

Figure 4. Inverse agonist activity of different CysLT$_{1}$R antagonists in cells expressing the WT with G$_{aq}$ protein or N106A mutant CysLT$_{1}$ receptors
COS-7 cells were incubated for 20 min with the indicated compounds, and inositol phosphate accumulation was measured as described in Methods. (A) Effect of inverse agonists on the wild-type CysLT1R receptor with Gαq basal inositol phosphate production (p<0.05) (B) Effect of inverse agonists on the N106A mutant receptor basal inositol phosphate production (*p<0.05). Concentrations used are as in Figure 3. The results are expressed as the means ± s.e. of three independent experiments, each done in triplicate.

Figure 5. Concentration-dependent effects of ligands with inverse agonist activity
Inverse agonist activity was examined in transiently transfected COS-7 cells. Effect of graded concentrations of selected inverse agonists on cells expressing (A) the wild-type CysLT1R receptor and Gαq or (B) the N106A mutant receptor. The results are expressed as the means ± s.e. of three independent experiments, each done in triplicate.

Figure 6. Effect of a neutral antagonist on inverse agonist activity
Cells expressing the N106A mutant receptor were untreated (unstimulated) or pre-treated with medium or REV5901, then exposed to the inverse agonists Montelukast, MK571 or Zafirlukast. Inositol phosphate accumulation was then measured as described in “Methods”. Concentrations of ligands used are as in Figure 3. The results are expressed as the means ± s.e. of three independent experiments, each done in triplicate. ** p<0.01, * p<0.05.

Figure 7. Sequestration of the CysLT1 receptors exposed to selected antagonists
Sequestration of WT CysLT1 and N106A receptors was measured following a 45 minute stimulation with LTD4 (5x10⁻⁷M) or selected LTD4 antagonists at concentrations described in
Figure 3. The cells were then incubated with anti-cMyc antibody, followed by a FITC-conjugated goat anti-mouse antibody. Cell-surface receptor expression was then determined on a FACScan flow cytometer. The results are expressed as the means ± se of three independent experiments. ** p<0.01, * p<0.05.
Table I

*Inositol phosphate production by different receptor combinations*

Inositol phosphate accumulations were determined as described under experimental procedures on transiently transfected COS-7 cells. Results are expressed as a ratio of IPs produced by indicated receptor combinations over IPs produced by the WT receptor stimulated with LTD$_4$ (5$\times$10$^{-7}$M). The results are the means ± s.e. of three independent determinations.

<table>
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<th>IP ratio</th>
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<tr>
<td>WT + LTD$_4$ 5$\times$10$^{-7}$M</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>WT + G$_{\alpha}q$</td>
<td>1.20 ± 0.05</td>
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<tr>
<td>N106A</td>
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<td>I232R</td>
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Table II

*Ligand binding of the wild-type and mutant receptors*

Binding parameters were determined as described under experimental procedures on transiently transfected COS-7 cells. Dissociation constants (K<sub>i</sub>-K<sub>d</sub>) are expressed in nM. The results are the means ± s.e. of four independent determinations.

<table>
<thead>
<tr>
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<th>WT (nM)</th>
<th>N106A (nM)</th>
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<tr>
<td>LTD&lt;sub&gt;4&lt;/sub&gt;</td>
<td>25 ± 2</td>
<td>16 ± 4</td>
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<tr>
<td>MK571</td>
<td>41 ± 2</td>
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<td>Zafirlukast</td>
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<tr>
<td>Montelukast</td>
<td>41 ± 1</td>
<td>24 ± 3</td>
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Figure 1

The graph shows the binding of $^3$H-LTD$_4$ (expressed as a percentage of specific binding) against the logarithm of LTD$_4$ concentration (M). Two different conditions are depicted: WT and N106A.
Figure 5