Inverse Agonist Activity of Selected Ligands of the Cysteinyl-Leukotriene Receptor 1

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ABSTRACT

Cysteinyl leukotrienes (CysLTs) are associated with several inflammatory processes, including asthma. Due to this association, considerable effort has been invested in the development of antagonists to the CysLT receptors (CysLT1R). Many 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 coexpressed with the Gq 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 3-[[3-[(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)propionic acid (MK571), the basal inositol phosphate production was reduced by 53 ± 6, 44 ± 3, and 54 ± 4%, respectively. On the other hand, 6(R)-(4-carboxyphenylthio)-5(R)-hydroxy-7(E),9(E),11(Z),14(Z)-eicosatetraenoic acid (BayU9773) and 1-[2-hydroxy-3-propyl-4-[(1H-tetrazole-5-YL)-butoxy]-phenyl ethanone] (LY171883) acted as partial agonists and α-pentyl-3-[2-quinoilnethyl]benzyl alcohol (REV 5901) as a neutral antagonist. However, in cells expressing CysLT1R and Gq, 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 signaling of the CysLT1 receptor indicated limited signaling through Gq-linked pathways (Lynch et al., 1999), probably via Gq11 as previously suggested by the 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; Leffkowitz 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 mutants in the platelet-activating factor receptor and other receptors (Parent et al., 1996a). In the CysLT1R, the substi-
tutions of isoleucine 232 of the third intracellular loop to an arginine (I232R) and of asparagine 106 of the second transmembrane segment to alanine (N106A) stabilize the active form and leads, in COS-7 cells, to a significant increase in the basal production of inositol phosphates (IPs). In previous studies, we and others demonstrated that coexpression of WT receptor and G proteins induced higher 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 overexpressed 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 to define which ones have inverse agonist, partial agonist, or neutral antagonist (no effect on activation) properties.

By expressing either the combination WT/G₁₂α 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.

Materials and Methods

Materials. Montelukast (1 μM) was obtained from Merck Frosst (Pointe-Claire, QC, Canada) and Zafirlukast (1 μM) from AstraZeneca (Lund, Sweden). BayU9773 (1.5 μM), LY171883 (6 μM), REV9501 (10 μM), and MK571 (1 μM) were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Cell Culture and Transfections. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium high glucose (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Sigma Chemical, Oakville ON, Canada) and transfected using FuGENE-6 (Roche Diagnostics, Mississauga, ON, Canada). Cells were plated at a density of 2.5 × 10⁵ cells/well in six-well plates and the next day, transfected exactly as instructed in Roche Diagnostic’s protocol, using 2 μl of FuGENE-6 and 1 μg of DNA. Experiments were carried out 2 days after transfection.

Radioligand Binding Assay. [³H]LTD₄ (PerkinElmer Life Sciences, Boston, MA) binding reactions were performed, as described previously by Sarau et al. (1999) with some modifications. Reactions were carried out on whole COS-7 cells at pH 7.4 transfected with DNA encoding a c-myc 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 glucose, and 10 mM cysteine, pH 7.4). The binding assays were done on 5 × 10⁴ cells in a total volume of 0.25 ml of the same buffer, containing 1 nM [⁴H]LTD₄, at room temperature 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 α₁ subunit of the human trimeric Gq protein and a c-myc epitope-tagged (N terminus) WT or a mutant receptor (I232R or N106A). The next day, cells were labeled for 18 to 24 h with 5 μCi/ml [³H]-myo-inositol (Amersham Biosciences Inc., Piscataway, NJ) in Dulbecco’s modified Eagle’s medium (high glucose, without inositol) (Invitrogen, Burlington, ON, Canada). After labeling, 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 were incubated at 37°C for 55 min. The reaction was terminated 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, Hercules, CA) as described previously (Parent et al., 1996b). [³H]-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 c-myc 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-c-Myc 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, West Grove, PA). Receptor expression on the cell surface was then evaluated using an FACScan flow cytometer (BD Biosciences, Oakville, ON, Canada).

Statistical Analysis. Statistical analyses were done using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA) following the methods and equations described in Motulsky and Christopoulos linear and nonlinear 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 cotransfected with the cDNA of the human CysLT₁R and G₁₂α. Other members of the α₁ family (G₁₃α, G₁₄α, and G₁₅α) were tested, but their effect on basal inositol phosphate production was lower than that of G₁₂α (data not shown). 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, and the mutant 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 [3H]LTD4 could be observed. The IC50 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 (data not shown). However, the I232R mutant had very high basal activity, as shown by IP production (Table 1), producing more than 3 times the level of IPs than the WT receptor. The IC50 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 expressed levels of cell surface proteins, as examined by flow cytometry with an anti-Myc antibody (data not shown). However, the I232R mutant had very high basal activity, as shown by IP production, whereas the basal production of WT/Gaq 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 transiently transfected COS-7 cells. Figure 2, A and B, shows competition isotherms for the WT and N106A CysLT1 receptors, respectively. The Kd and Kf values of LTD4, MK571, Zafirlukast, and Montelukast in competition with [3H]LTD4 are represented in Table 2. These compounds are all characterized as CysLT1R ligands, and they effectively interfered with [3H]LTD4 binding to both WT and N106A mutant receptors. Their efficacy in blocking LTD4-stimulated inositol phosphate production in cells expressing the WT CysLT1R, R, and Gαq was also examined (Fig. 3). Concentrations described in literature for their maximal efficacy were used for each experiment. Montelukast (1 μM), MK571 (1 μM), BayU9773 (1.5 μM), Zafirlukast (1 μM), LY171883 (6 μM), and REV5901 (10 μM) 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 + Gαq 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 + Gαq receptor corresponding to a reduction of approximately 20 to 25% of basal IPs (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 (Fig. 4B).

| Table 1 | Inositol phosphate production by different receptor combinations
<table>
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<tr>
<td>WT + LTD4 5 x 10^-7 M</td>
<td>1.00 ± 0.00</td>
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<tr>
<td>WT + Gαq</td>
<td>1.20 ± 0.05</td>
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<tr>
<td>N106A</td>
<td>1.30 ± 0.11</td>
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<td>I232R</td>
<td>3.20 ± 0.18</td>
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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; Shroyack et al., 1998; Spadoni et al., 1998). Here, we report that molecules known to antagonize diverse responses to LTD4 can also inhibit spontaneous activity of CysLT1R.

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, equilibrium between active and inactive conformational states in the absence of agonist (Leff, 1993; Milligan et al., 1995; Leurs et al., 1998). Compounds displaying inverse agonism should have a higher affinity for the inactive state compared with 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 Gq 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.

### Table 2

<table>
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<tr>
<th>Ligand binding of the wild-type and mutant receptors</th>
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<td>Binding parameters were determined as described under Materials and Methods on transiently transfected COS-7 cells. Dissociation constants (Ki and Kd) are expressed in nanomolar concentration. The results are the means ± S.E. of four independent determinations.</td>
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<td>WT</td>
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<td>LTD4</td>
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<td>Montelukast</td>
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### Fig. 2

Effect of CysLT1R antagonists on [3H]LTD4 binding to wild-type CysLT1 receptor. Binding reactions were performed on COS-7 cells transiently expressing WT CysLT1 (A) or N106A receptor (B) in presence of various concentrations of indicated antagonists as described under Materials and Methods. The results are expressed as the means of three independent experiments, each done in duplicate.

### Fig. 3

Efficiency of indicated antagonists on blocking LTD4-induced inositol phosphate production in COS-7 cells overexpressing wild-type CysLT1R and Gq. Inositol phosphate production was measured after a 20-min pretreatment with antagonists, followed by stimulation with LTD4 (5 × 10^{-7} M). The concentrations of antagonists used were Montelukast (1 µM), MK571 (1 µM), BayU9773 (1.5 µM), Zafirlukast (1 µM), LY171883 (6 µM), and REV5901 (10 µM). The results are expressed as the means ± S.E. of three different independent experiments, each done in triplicate.

### Fig. 4

Inverse agonist activity of different CysLT1R antagonists in cells expressing the WT with Gq protein or N106A mutant CysLT1 receptors. COS-7 cells were incubated for 20 min with the indicated compounds, and inositol phosphate accumulation was measured as described under Materials and Methods. A, effect of inverse agonists on the wild-type CysLT1R receptor with Gq 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 Fig. 3. The results are expressed as the means ± S.E. of three independent experiments, each done in triplicate.
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 on 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 that 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 CCK3 receptor (Roeftger 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 desensitization and up-regulation of receptor expression (Smit et al., 1996; Lee et al., 1997; Zhu et al., 2000). However, this would not apply.
under our conditions because our treatment with inverse agonists was relatively short. The increased cell surface expression could be due to the stabilization of the receptor, either intracellularly 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). Because 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 β2-adrenoceptor 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 because 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 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 signaling. 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 up-regulation 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, in 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 speculate 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 that could induce short- or long-term state of unresponsiveness without inducing an effector cascade.

References


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