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*J Immunol* 2010; 184:2219-2225; Prepublished online 18 January 2010;
doi: 10.4049/jimmunol.0900071
http://www.jimmunol.org/content/184/4/2219

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Concentration-Dependent Noncysteinyl Leukotriene Type 1 Receptor-Mediated Inhibitory Activity of Leukotriene Receptor Antagonists

Grzegorz Woszczek,*+1 Li-Yuan Chen,*+1 Sara Alsaaty,* Sahrudaya Nagineni,* and James H. Shelhamer*

The use of cysteinyl leukotriene receptor antagonists (LTRAs) for asthma therapy has been associated with a significant degree of interpatient variability in response to treatment. Some of that variability may be attributable to noncysteinyl leukotriene type 1 receptor (CysLT₁)–mediated inhibitory mechanisms that have been demonstrated for this group of drugs. We used a model of CysLT₁ signaling in human monocytes to characterize CysLT₁-dependent and -independent anti-inflammatory activity of two chemically different, clinically relevant LTRAs (montelukast and zafirlukast). Using receptor-desensitization experiments in monocytes and CysLT₁-transfected HEK293 cells and IL-10– and CysLT₁ small interfering RNA–induced downregulation of CysLT₁ expression, we showed that reported CysLT₁ agonists leukotriene D₄ and UDP signal through calcium mobilization, acting on separate receptors, and that both pathways were inhibited by montelukast and zafirlukast. However, 3-log greater concentrations of LTRAs were required for the inhibition of UDP-induced signaling. In monocytes, UDP, but not leukotriene D₄, induced IL-8 production that was significantly inhibited by both drugs at micromolar concentrations. At low micromolar concentrations, both LTRAs also showed that reported CysLT₁ agonists leukotriene D₄ and UDP signal through calcium mobilization, acting on separate receptors, and that both pathways were inhibited by montelukast and zafirlukast. However, 3-log greater concentrations of LTRAs were required for the inhibition of UDP-induced signaling. In monocytes, UDP, but not leukotriene D₄, induced IL-8 production that was significantly inhibited by both drugs at micromolar concentrations. At low micromolar concentrations, both LTRAs also inhibited calcium ionophore-induced leukotriene (leukotriene B₄ and leukotriene C₄) production, indicating 5-lipoxygenase inhibitory activities. We report herein that montelukast and zafirlukast, acting in a concentration-dependent manner, can inhibit non–CysLT₁-mediated proinflammatory reactions, suggesting activities potentially relevant for interpatient variability in response to treatment. Higher doses of currently known LTRAs or new compounds derived from this class of drugs may represent a new strategy for finding more efficient therapy for bronchial asthma. *The Journal of Immunology, 2010, 184: 2219–2225.

Leukotrienes have been implicated in the pathophysiology of bronchial asthma. Drugs inhibiting leukotriene signaling, such as cysteinyl leukotriene receptor antagonists (LTRAs) (montelukast, pranlukast, and zafirlukast), were shown to be effective in treating this disease (1, 2). However, their use has been associated with a significant degree of interpatient variability in response to treatment. In clinical trials, 20–50% of patients receivingLTRAs could be classified as responders, showing a great heterogeneity in response to these drugs, which affects the effectiveness of treatment (3, 4). Much of that variability may be attributable to genetic variations; several studies reported that genetic polymorphisms in genes encoding key proteins in the leukotriene pathway might influence the response to LTRAs (5, 6). Another cause of variability may be associated with the pharmacokinetic characteristics of the drugs used and the final individual compound plasma concentrations obtained during treatment.

All LTRAs act on the cysteinyl leukotriene type 1 receptor (CysLT₁) and by competitive antagonism at this receptor are believed to be responsible for the control of airway inflammation, bronchoconstriction, and remodeling (7, 8). However, anti-inflammatory activity of LTRAs independent of CysLT₁ antagonism was recently suggested. Montelukast reduced the expression of urokinase plasminogen activator receptor and the secretion of metalloproteinase-9 in human eosinophils in vitro (9), and it inhibited TNF-α–mediated IL-8 expression in U937 cells through mechanisms distinct from CysLT₁ antagonism (10). Interestingly, it was shown that montelukast may have a novel inhibitory effect on 5-lipoxygenase (5-LO) activity (11) and transport of leukotrienes by the multidrug resistance protein ABCC4 (12), suggesting a broader mechanism of action for this drug. Non–CysLT₁-related mechanisms of LTRA actions might present another level of variability in the response to treatment in patients with asthma. Some of these non–CysLT₁-related activities of LTRAs may be compound specific or may depend on drug concentration or the presence of a particular inflammatory pathway in patients with asthma; therefore, clinically significant effects of treatment may be observed in some, but not all, treated subjects.

We previously showed that CysLT₁ is the predominantly expressed leukotriene receptor in human endothelial monocytes and that leukotriene D₄ (LTD₄), acting through CysLT₁, can induce the activation and chemotaxis of these cells (13). In the current study, we used this model of CysLT₁ signaling in human monocytes to characterize the CysLT₁-dependent and -independent inhibitory activity of two chemically different, clinically relevant LTRAs (montelukast and zafirlukast) and to define the pathways of their inhibitory action.

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Received for publication January 9, 2009. Accepted for publication December 16, 2009.

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Abbreviations used in this paper: Con, control oligonucleotides; cysLT, cysteinyl leukotriene; CysLT₁, cysteinyl leukotriene type 1 receptor; CysLT₂, cysteinyl leukotriene type 2 receptor; GPCR, G-protein coupled receptor; 5-LO, 5-lipoxygenase; LTD₄, leukotriene B₄; LTC₄, leukotriene C₄; LTB₄, leukotriene D₄; LTRA, cysLT receptor antagonist; Mon, montelukast; MRS2578, P2Y₆ inhibitor; siRNA, small interfering RNA; Zaf, zafirlukast.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900071
Materials and Methods

LTD₄, montelukast, and zafirlukast (Cayman Chemical, Ann Arbor, MI), calcium ionophore A23187 (EMD Chemicals, Gibbstown, NJ), UDP, P2Y₆ inhibitor (MR52578), DMSO (all from Sigma-Aldrich, St. Louis, MO), and human rIL-10 (R&D Systems, Minneapolis, MN) were obtained from the manufacturers.

Cell culture

Human elutriated monocytes from healthy donors were obtained by an institutional review board-approved protocol from the National Institutes of Health Blood Bank (Bethesda, MD), resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 2 mmol/l-glutamine (all from Invitrogen, Carlsbad, CA), and allowed to rest overnight before experiments at 37˚C in a humidified 5% CO₂ incubator.

Human embryonic kidney (HEK293) cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS.

Calcium mobilization assay

Calcium mobilization experiments were conducted using a FLIPR Calcium 3 assay kit (Molecular Devices, Sunnyvale, CA), according to the manufacturer’s instructions. Cells (2 × 10⁶ cells/well) were plated into poly-l-lysine-coated 96-well plates and incubated in RPMI 1640 supplemented with 10 mmol/l HEPES and FLIPR 3 assay reagent. After incubation for 1 h at 37˚C, fluorescence was measured every 4 s using the FlexStation (Molecular Devices).

HEK293 cells were grown in 75-ml flasks and transfected with empty pcDNA 3.1 vector or CysLT₁ expression vector (Missouri University of Science and Technology cDNA Resource Center, Rolla, MO) using Lipofectamine 2000 (Invitrogen) in serum-free medium (Opti-MEM I, Invitrogen), transferred to poly-l-lysine-coated 96-well plates after 24 h, and used for calcium mobilization experiments after a 24-h incubation of control.

CysLT₁, cysteinyl leukotriene type 2 receptor, and P2Y₆ knockdown

Silencer Select predesigned small interfering RNA (siRNA) (CysLT₁): 5’-G-GAAGCGGUGCCUAUtt-3’; cysteinyl leukotriene type 2 receptor [CysLT₂]: 5’-GCACAAUUGAAUUCAACAA-3’; P2Y₆: 5’-GAAGCCUC- ACCAAAAACUAUt-3’) and Silencer Select Negative Control siRNA (Ambion, Austin, TX) were used for CysLT₁, CysLT₂, and P2Y₆ knockdown experiments. Elutriated monocytes (5 × 10⁶) were nucleofected with 4 µg negative control or specific siRNA using a Human Monocyte Nucleofector kit (Axama, Cologne, Germany), according to the manufacturer’s protocol. After 24 h, media were replaced, and cells were used for functional studies.

Real-time PCR

Total RNA was extracted from cells using QIA Shredder columns and RNeasy mini kit and was treated with DNase (Qiagen, Valencia, CA, USA). mRNA expression for selected genes was measured using real-time PCR performed on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) using the following commercially available probe and primers set (Applied Biosystems): CysLT₁, Hs00272624_s1; CysLT₂, Hs00252658_s1; and P2Y₆, Hs00602548_m1. Reverse transcription and PCR were performed using an RT kit and TaqMan Universal PCR master mix (Applied Biosystems), according to the manufacturer’s directions. Relative gene expression was normalized to GAPDH transcripts and calculated as a fold-change compared with control.

ELISA

For IL-8 determination, monocytes (2 × 10⁶) were stimulated with LTD₄ (100 nmol/l) or UDP (100 µmol/l) for 2 and 6 h in the presence or absence of inhibitors. Supernatants were stored at −20°C until analysis by commercial Quantikine ELISA (R&D Systems) for IL-8.

Determination of leukotriene B₂, leukotriene C₄, and PGE₂ synthesis

Monocytes (1 × 10⁶) were resuspended in culture medium without FBS; preincubated with different concentrations of montelukast, zafirlukast, or vehicle control (DMSO); and stimulated with calcium ionophore A23187 (1 µmol/l) or DMSO for 30 min. Supernatants were centrifuged and frozen at −80°C for leukotriene measurement. For intracellular leukotriene determination, elutriated monocytes (2 × 10⁶) were used, reactions were stopped by the addition of cold PBS, and cells were collected by centrifugation at 4°C. Cells were lysed by freezing (three times), followed by lipid purification using an solid phase extraction (C-18) cartridge (Cayman Chemical), according to the manufacturer’s protocol. Purified samples were resuspended in enzyme immunoassay buffer and assayed directly. The concentration of leukotriene B₂ (LTB₂), leukotriene C₄ (LTC₄), and PGE₂ produced spontaneously and after stimulation was assayed by means of competitive enzyme immunosorbent assay (Cayman Chemical), according to the manufacturer’s protocol.

Statistical analysis

Data were analyzed by one-way ANOVA or paired and unpaired Student’s t tests, as appropriate. Differences were considered significant when p < 0.05.

Results

LTRAs inhibit LTD₄- and UDP-induced calcium flux

It was suggested that CysLT₁ has a dual cysteinyl leukotriene (cysLT)/UDP specificity (14). To investigate a role for LTRAs in CysLT₁-mediated reactions, the responsiveness of human monocytes to LTD₄ and UDP was evaluated by intracellular calcium flux measurement. As shown in Fig. 1, monocytes responded in a concentration-dependent manner to LTD₄ and UDP. However, the concentration of UDP that was most effective at inducing calcium flux was 3-log greater (10⁻⁶ mol/l) than the most effective concentration of LTD₄ (10⁻⁷–10⁻⁶ mol/l). CysLT₁ antagonists (montelukast and zafirlukast) were used to determine whether LTD₄ similarly inhibit UDP and LTD₄ signaling in monocytes. Both antagonists inhibited (Fig. 2) LTD₄- and UDP-induced calcium flux in monocytes in a concentration-dependent manner. However, as only low nanomolar concentrations of montelukast and zafirlukast were required to fully inhibit LTD₄-induced calcium flux, much higher (10⁻⁵–10⁻⁴ mol/l) concentrations of antagonists were required to inhibit UDP-induced calcium changes.

LTD₄ and UDP signal through separate receptors in monocytes

To further characterize the responsiveness of human monocytes to LTD₄ or UDP, the submaximally effective concentrations of LTD₄ (10⁻⁷ mol/l) and UDP (10⁻⁴ mol/l) were used to analyze the pattern of calcium signaling and desensitization. In monocytes, LTD₄ induced a rapid increase in intracellular calcium levels that relatively quickly decreased to baseline levels (Fig. 3A). UDP also rapidly increased intracellular calcium levels; however, these were sustained for only 10–20 s and then decreased to baseline. The different patterns of calcium changes induced by UDP compared with LTD₄ suggested that these compounds could activate different receptors or signaling pathways. In agreement with the above observation, stimulation with LTD₄ and UDP signal through separate receptors in monocytes.

![FIGURE 1](https://www.jimmunol.org/)}
LTD$_4$ ($10^{-7}$ mol/l) potently desensitized monocytes to subsequent stimulation with the same concentration of LTD$_4$ ($10^{-7}$) (as expected for homologous desensitization), but it did not have any effect on subsequent UDP exposure, suggesting again that UDP could act through a separate receptor (not CysLT$_1$). When cells were stimulated with UDP, full desensitization of calcium signaling was observed for the subsequent UDP exposure (homologous desensitization) and partial inhibition of signaling to subsequent LTD$_4$ stimulation. To determine whether LTD$_4$ and UDP could activate CysLT$_1$, HEK293 cells were transiently transfected with the human CysLT$_1$ construct. HEK293 cells did not express CysLT$_1$ mRNA and, when transfected with empty pcDNA 3.1 vector, did not show calcium responses after LTD$_4$ stimulation up to $10^{-6}$ mol/l concentration (data not shown). In cells transfected with CysLT$_1$ (Fig. 3B), LTD$_4$ induced calcium flux with a pattern similar to that observed in monocytes, and full homologous desensitization to LTD$_4$ was present. However, empty vector and CysLT$_1$-transfected HEK293 cells responded to UDP with potent calcium flux; the most effective concentration was $10^{-7}$ mol/l. When the desensitization experiments were carried out, a similar level of homologous desensitization to UDP was observed in empty control and CysLT$_1$-transfected cells. Interestingly, the same pattern of heterologous desensitization as in monocytes was identified in CysLT$_1$-transfected cells; partial desensitization by UDP to subsequent LTD$_4$ stimulation and no desensitization by LTD$_4$ to subsequent UDP exposure. 

All of these data suggested that UDP induced calcium flux through a separate receptor, not CysLT$_1$. It was shown that UDP can induce calcium flux in several cell systems acting through nucleotide G-protein-coupled receptors (GPCRs), with P2Y$_6$ being the most selective for UDP. We confirmed the presence of P2Y$_6$ mRNA in monocytes, but the level of expression of P2Y$_6$ mRNA in HEK293 cells was very low (data not shown), suggesting that UDP could activate calcium flux through this or other nucleotide receptors in these cells. UDP (100 μM) induced a calcium flux in untransfected HEK cells that was not inhibited by the P2Y$_6$ inhibitor MRS2578 (5 μM). When used at micromolar concentrations, MRS2578 inhibited UDP- and LTD$_4$-induced calcium flux in monocytes in a concentration-dependent manner (Fig. 4). We previously reported that IL-10 downregulates CysLT$_1$ expression and signaling in human monocytes (15). Monocytes were exposed to IL-10 (20 ng/ml) overnight to downregulate CysLT$_1$, and calcium flux to LTD$_4$ and UDP was evaluated. IL-10 downregulated CysLT$_1$ mRNA (measured by real-time PCR) and inhibited LTD$_4$-induced calcium flux, whereas UDP signaling was not significantly affected (Fig. 5A), suggesting that UDP does not signal through CysLT$_1$ in monocytes. To further confirm this observation, CysLT$_1$ expression was specifically downregulated by siRNA, as previously described (15), and calcium flux was measured in response to different concentrations of LTD$_4$ and UDP. As expected, in CysLT$_1$ siRNA-treated cells, LTD$_4$-induced calcium flux was significantly inhibited, in contrast to UDP-induced signaling, which remained similar in CysLT$_1$ siRNA-treated cells and control cells (Fig. 5B). Treatment of cells with siRNA directed at CysLT$_2$, reduced CysLT$_2$ mRNA by 87%, but it did not reduce LTD$_4$- or UDP-induced calcium flux (data not shown). To analyze whether UDP signals through P2Y$_6$ in monocytes, P2Y$_6$ was knocked down using siRNA, and the response to LTD$_4$ and UDP was measured. P2Y$_6$ mRNA was knocked down by 85% (four measurements). UDP-induced calcium flux was inhibited in P2Y$_6$ siRNA-treated cells, whereas LTD$_4$-induced signaling was not affected (Fig. 6), showing that P2Y$_6$ may be one of the target receptors for UDP-induced signaling in monocytes.

All of these data suggest that in human monocytes, LTD$_4$ and UDP activate separate receptors, LTD$_4$ induces calcium flux through CysLT$_1$ and UDP signals at least in part through P2Y$_6$.

**LTRA inhibits UDP-induced IL-8 production**

It was reported that UDP mediates IL-8 production in monocytic THP-1 cells (16). To determine whether LTRAs may inhibit other UDP-induced functions, in addition to intracellular calcium flux,
monocytes were stimulated with LTD₄ or UDP, and the synthesis of IL-8 was measured as described in Materials and Methods. UDP, but not LTD₄, induced IL-8 production in a time-dependent manner (Fig. 7A) that was significantly inhibited by the P₂Y₆ inhibitor MRS2578 (10 μmol/l). Montelukast and zafirlukast also inhibited IL-8 production in a concentration-dependent manner (Fig. 7B). These data further confirm that LTD₄ and UDP signal differently in human monocytes and that LTRAs may significantly affect the UDP-induced activation of these cells. It was recently reported that CysLT₁ knockdown inhibited LTD₄- and UDP-induced chemokine generation in human mast cells (17). In contrast, we did not observe significant changes in chemokine (IL-8) generation in CysLT₁ siRNA-treated monocytes compared with control siRNA-treated cells (data not shown). This suggests that UDP and cysteine-containing agonists may signal differently in different types of cells (mast cells and monocytes), probably reflecting differences in GPCR expression.

**LTRAs inhibit leukotriene production**

To establish whether LTRAs may affect 5-LO activity and leukotriene production, monocytes were stimulated with calcium ionophore A23187, and synthesis of LTB₄ and LTC₄ was measured. Stimulated monocytes produced significant levels of both leukotrienes, which were inhibited by pretreatment with montelukast (Fig. 8A) and zafirlukast (Fig. 8B) in a concentration-dependent fashion. Both antagonists (5 μmol/l) fully inhibited leukotriene production, suggesting that 5-LO could be directly affected by the treatment. To exclude the possibility that LTRAs affect arachidonic acid synthesis by phospholipase A₂, PGE₂, as a product of the cyclooxygenase pathway of arachidonic acid cascade, was measured in the same experiments. Neither montelukast nor zafirlukast (5 mol/l) inhibited calcium ionophore-induced PGE₂ generation (Fig. 8D). To further analyze whether LTRAs inhibit the production of leukotrienes and not transport out of the cell, monocytes were stimulated with calcium ionophore, and intracellular levels of LTB₄ and LTC₄ were measured as described in Materials and Methods. Calcium ionophore stimulation produced detectable intracellular levels of both leukotrienes (Fig. 8C), which were inhibited by pretreatment with montelukast and zafirlukast, indicating that LTRAs may significantly downregulate the production of leukotrienes in human monocytes.

**Discussion**

We presented evidence herein that so-called “selective CysLT₁ antagonists” montelukast and zafirlukast possess concentration-dependent non-CysLT₁-mediated inhibitory activities in primary human monocytes. Both drugs effectively inhibited LTD₄-induced signaling at the nanomolar concentration range, in agreement with CysLT₁ antagonist activity reported in previous studies (18, 19). Interestingly, montelukast and zafirlukast, acting at low micromolar concentrations, also inhibited LTC₄ and LTB₄ synthesis, probably through a direct inhibition of 5-LO. It was suggested that montelukast may directly inhibit 5-LO activity, binding to an undefined allosteric site on 5-LO (11). In our study, montelukast and zafirlukast similarly inhibited leukotriene synthesis, but not PGE₂ generation, suggesting that this 5-LO inhibitory activity is not specific for montelukast; it can be applied to other LTRAs as well. The next proinflammatory pathway, inhibited in our study by both compounds at higher micromolar concentrations, is UDP-induced signaling and IL-8 production. Extracellular nucleotides, such as UTP and UDP, were shown to activate phospholipase C and intracellular calcium mobilization in differentiated U937 cells through purine P₂Y receptors; these effects were inhibited by micromolar concentrations of montelukast and pranlukast (20). We validated these observations...
in a human primary cell system, and we showed that such an inhibition of signaling may have functional consequences for cell activation, measured at the level of UDP-induced IL-8 production. We report herein that two chemically different LTRAs might possess, in addition to CysLT1 antagonism, anti-inflammatory activities in primary human cells. The fact that these additional inhibitory activities are strongly related to the drug concentration might have potential clinical relevance. LTRAs, in contrast to many other drugs, are believed to require membrane transporters for their absorption after oral administration (21–23). In addition, other features, such as strong binding to plasma albumin (99%) and extensive hepatic metabolism, suggest that the bioavailability of those drugs might be affected by pharmacogenetic and pharmacokinetic factors, resulting in variability of plasma drug concentration and response to treatment. Although there are no published population-based data demonstrating pharmacogenetic influence on LTRA plasma concentrations, case reports showed significant variations in montelukast plasma levels in healthy subjects (24). In addition, it was recently shown that genetic variation of OATP2B1, a membrane transport protein, was associated with variable montelukast plasma concentrations and variable response to treatment in patients with asthma (25). The maximum plasma concentration after orally administered montelukast, 10 mg, ranged from 0.57 to 0.63 μmol/l in healthy female and male subjects, respectively (26); it reached 0.80 μmol/l following administration of a 10-mg chewable tablet in adults and a 5-mg chewable tablet in 9- to 14-y-old children (27). These concentrations are very close to the concentrations of montelukast and zafirlukast required for leukotriene synthesis inhibition (1–5 μmol/l) in our study. Bearing in mind potential genetically driven variability in drug absorption and metabolism, it is plausible to consider that even after the administration of a standard dose of LTRAs in some patients, inhibition of the leukotriene synthesis pathway might be observed in addition to CysLT1 antagonism. The clinical relevance of additional inhibitory activity of LTRAs on 5-LO has not been addressed. In a placebo-controlled, dose-ranging study of montelukast in patients with asthma, doses from 10–200 mg/d were analyzed; no dose-response relationship for asthma symptom control and spirometry was observed (28). However, in patients treated with 100 and 200 mg [calculated maximum plasma concentration >3 and 7 μmol/l, respectively (29)], the morning and evening peak expiratory flow rates showed nearly twice the improvement compared to patients receiving lower doses, suggesting that higher doses of this drug could have an

FIGURE 6. Knockdown of P2Y6 did not affect LTD4-induced signaling in monocytes. Monocytes were nucleofected with P2Y6 siRNA or negative control oligonucleotides (control), as described in Materials and Methods, cultured for 24 h, and calcium flux was measured in response to different concentrations of UDP (A, B) or LTD4 (C, D). Representative traces of calcium flux from one of three separate experiments, each with similar results, are shown.

FIGURE 7. Montelukast and zafirlukast inhibit UDP-induced IL-8 production in monocytes. A. Monocytes were preincubated with MRS2578 or vehicle control for 30 min and stimulated with LTD4 (100 nmol/l) or UDP (100 μmol/l). Supernatants were collected after 2 and 6 h, and IL-8 expression was measured. B. Monocytes were preincubated with different concentrations of LTRAs, stimulated with UDP, and IL-8 expression was measured after 6 h. Data are presented as the percentage of UDP-induced IL-8 production from cells pretreated with vehicle control. Means ± SD from three separate experiments are presented. *p < 0.001, Student t test.
additional impact on asthma treatment. The inhibition of UDP signaling by LTRAs required higher micromolar concentrations (10–100 μmol/l) in our study. The maximum plasma concentration exceeded 25 μmol/l after the oral administration of 800 mg of montelukast, a dose that was still well tolerated (29); however, the clinical relevance of such doses has not been tested. It was shown that LTRAs may also affect other non–CysLT1-mediated pathways in human primary cells. Montelukast inhibited eosinophil protease activity (9), and pranlukast suppressed LPS-induced IL-6 production in PBMCs (30); both effects were observed at micromolar drug concentrations. All of these data strongly suggest that in addition to CysLT1 antagonism, other proinflammatory pathways may be affected by LTRA treatment and, depending on plasma drug concentration, variable responses to treatment may be expected.

It was suggested that CysLT1 expressed on human mast cells is also a pyrimidinergic receptor and that it responds to UDP stimulation in addition to cysLTs (14, 31). In our study, human monocytes responded to LTD4 and UDP, but with characteristics suggesting that UDP acts through separate receptors. First, 3-log greater concentrations (10⁻⁴ mol/l) of UDP were required to submaximally activate calcium flux in monocytes than reported in the study by Mellor et al. (14). Although UDP signaling in monocytes was inhibited by LTRAs, much greater concentrations of LTRAs were needed. Almost every GPCR studied undergoes desensitization following agonist stimulation, an important process that is crucial for turning off the receptor-mediated signaling pathway. As expected, CysLT1 underwent LTD4-induced homologous desensitization in monocytes, but we did not observe desensitization of UDP-induced calcium flux subsequent to LTD4 treatment, a phenomenon that should be present when both agonists act on the same GPCR. A similar observation was reported in differentiated U937 cells (32); CysLT1 desensitization and trafficking were differentially regulated by LTD4 and extracellular nucleotides, including UDP. Capra et al. (32) showed that CysLT1 may be a target for extracellular nucleotide-induced heterologous desensitization, a potential feedback mechanism in inflammation. The presence of this heterologous desensitization mechanism was demonstrated for the first time in human primary cells in our study. UDP signals effectively through calcium flux acting on P2Y6 in human cells (33). Because human monocytes express mRNA for CysLT1 and P2Y6, we downregulated CysLT1 expression by IL-10 preincubation or specific CysLT1 siRNA to further support the observation that UDP does not signal through CysLT1 in monocytes; we noted a specific decrease in LTD4-induced signaling, without an effect on UDP-induced calcium mobilization. Knockdown of P2Y6 decreased UDP-induced calcium flux but did not affect LTD4-induced signaling. All of these data suggest that UDP is not a CysLT1 agonist in human cells, but it may signal through a different GPCR. In fact, a newly described cysLT receptor (GPR17) was shown to be a dual UDP/cysLT receptor (34).

We showed previously that GPR17 is not expressed in human monocytes (13), excluding this receptor as a potential target for UDP in monocytes. Interestingly, the presence of GPR17 was detected in mast cell line LAD2, an observation that might explain cysLT receptor-related signaling induced by UDP in mast cells (35). It was also suggested that additional, not-yet-characterized cysLT receptors might be expressed in mast cells (35).

The described inhibitory activity of LTRAs on the 5-LO pathway and extracellular nucleotide-induced signaling may be relevant for...
designing new asthma treatment strategies. LTD4, another product of the 5-LO pathway, was reported to contribute to the recruitment and activation of neutrophils and eosinophils and play an important role in asthma exacerbations (36). Extracellular nucleotides serve as a danger signal to alert the immune system of tissue damage. It was shown in a murine model that extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells (37). UDP may mediate microglial phagocytosis (38) and play a role in innate immune responses, but its significance for asthmatic inflammation is not known. Higher activations (38) and play a role in innate immune responses, but its significance for asthmatic inflammation is not known. Higher
dores of currently known LTRAs or new compounds derived from this class of drugs may represent a new strategy for finding more efficient therapy for bronchial asthma.

Disclosures
The authors have no financial conflicts of interest.

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