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CysLT₁ leukotriene receptor antagonists inhibit the effects of nucleotides acting at P2Y receptors

Liaman Mamedova^a, Valérie Capra^b, Maria Rosa Accomazzo^b, Zhan-Guo Gao^a, Silvia Ferrario^c, Marta Fumagalli^c, Maria P. Abbraccio^c, G. Enrico Rovati^b, and Kenneth A. Jacobson^{a,*}

^a Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

^b Section of Eicosanoid Pharmacology, Laboratory of Molecular Pharmacology, Department of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy

^c Laboratory of Molecular and Cellular Pharmacology of Purinergic Transmission, Department of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy

Abstract

Montelukast and pranlukast are orally active leukotriene receptor antagonists selective for the CysLT₁ receptor. Conversely, the hP2Y_{1,2,4,6,11,12,13,14} receptors represent a large family of GPCRs responding to either adenine or uracil nucleotides, or to sugar-nucleotides. Montelukast and pranlukast were found to inhibit nucleotide-induced calcium mobilization in a human monocyte-macrophage like cell line, DMSO-differentiated U937 (dU937). Montelukast and pranlukast inhibited the effects of UTP with IC₅₀ values of 7.7 and 4.3 μM, respectively, and inhibited the effects of UDP with IC₅₀ values of 4.5 and 1.6 μM, respectively, in an insurmountable manner. Furthermore, ligand binding studies using [³H]LTD₄ excluded the possibility of orthosteric nucleotide binding to the CysLT₁ receptor. dU937 cells were shown to express P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₃ and P2Y₁₄ receptors. Therefore, these antagonists were studied functionally in a heterologous expression system for the human P2Y receptors. In 1321N1 astrocytoma cells stably expressing human P2Y_{1,2,4,6} receptors, CysLT₁ antagonists inhibited both the P2Y agonist-induced activation of phospholipase C and intracellular Ca²⁺ mobilization. IC₅₀ values at P2Y₁ and P2Y₆ receptors were <1 μM. In control astrocytoma cells expressing an endogenous M3 muscarinic receptor, 10 μM montelukast had no effect on the carbachol-induced rise in intracellular Ca²⁺. These data demonstrated that CysLT₁ receptor antagonists interact functionally with signaling pathways of P2Y receptors, and this should foster the study of possible implications for the clinical use of these compounds in asthma or in other inflammatory conditions.

Keywords

Montelukast; Pranlukast; Purine receptors; Nucleotides; ATP; UDP

* Corresponding author. Tel.: +1 301 496 9024; fax: +1 301 480 8422. kajacobs@helix.nih.gov (K.A. Jacobson).

1. Introduction

Cysteinyl leukotrienes (CysLTs), LTC₄, LTD₄ and LTE₄, potent inflammatory mediators derived from arachidonic acid [1], are known to be very potent bronchoconstrictors and to play an important role in asthma and allergic rhinitis [2,3]. They have been also implicated in a number of inflammatory conditions including cardiovascular diseases, coronary artery disease, atherosclerosis and stroke [4–6] or in cardiovascular complications of inflammatory processes [7].

CysLTs trigger contractile and inflammatory processes through the specific interaction with cell surface receptors belonging to the rhodopsin family of the G protein-coupled receptor (GPCR) genes. Until now, two receptor subtypes have been cloned, namely CysLT₁ and CysLT₂ [8]. In particular, when the CysLT₁ receptor is expressed in recombinant systems it shows a preferential coupling to G_{q/11}, whereas when constitutively expressed it has been reported to activate both pertussis toxin (PTX)-sensitive and -insensitive G-proteins [8,9]. This was demonstrated in dimethyl sulfoxide-differentiated U937 (dU937) cells, an immortalized cell line known to constitutively express a high density of CysLT₁ receptors upon differentiation to monocytes/macrophages [10,11], CysLT₁ receptors respond to LTD₄ with a strong increase in cytosolic Ca²⁺ concentration ([Ca²⁺]_i) partially sensitive to PTX, and with the activation of the Ras-MAPK cascade totally dependent upon G_{i/o} [12]. These signaling effects were totally inhibited by various specific CysLT₁-receptor antagonists, and no CysLT₂ receptor mRNA was detected [13,14], thus indicating that in monocyte-macrophage like U937 cells LTD₄-induced responses can be totally ascribed to a CysLT₁ receptor. The promonocytic leukemia cell U937 was selected because closely related to the inflammatory cells responsible of many CysLT biological actions, and because monocyte/macrophages activation leads to the release of a wide spectrum of cytokines and chemokines that have key roles in all inflammatory diseases.

CysLT receptors belong to the group A subfamily of GPCRs and, in particular, to a cluster of receptors phylogenetically related to the purine P2Y receptors [15]. In addition to a number of orphan receptors this cluster includes receptors that respond to purinergic or pyrimidinergic nucleotides (P2Y receptors, see also below), proteases (F2R) and chemoattractants (FPR) [16,17]. The CysLT receptor nomenclature was originally based on the sensitivity to the antagonists, which include montelukast, zafirlukast, pranlukast, pobilukast and MK571 [9]. Montelukast (Singulair®), a potent CysLT₁ receptor antagonist belonging to the chemical class of quinolines [18], is an effective and well-tolerated preventative drug for asthma and allergic rhinitis in adults and children [19]. Pranlukast (Onon®, Azlaire®) belongs to the chemical class of benzopyrans [20] and is indicated for the prophylactic treatment of chronic bronchial asthma in pediatric and adult patients and is currently on the market only in Japan and South America [21]. CysLT₁ receptor antagonists have proven effective in various models of induced asthma, as well as in the treatment of chronic asthma. Preclinical and clinical studies have demonstrated that they differ in terms of potency and pharmacokinetics, but have similar pharmacodynamic profiles [22–24].

Conversely, P2Y receptors are GPCRs responsive to both adenine (ATP, ADP) and uracil (UTP, UDP) nucleotides, or to sugar nucleotides (UDP-glucose and UDP-galactose) [25]. Eight P2Y receptor subtypes are currently recognized: the P2Y_{1,2,4,6,11,12,13,14} receptors (ibidem). These receptors are widely distributed in human tissues, and important pathophysiological roles in brain function, haemostasis, regulation of blood pressure, inflammation and respiratory functions have been suggested [26]. Based on structural, phylogenetic properties and signaling coupling, a subclassification of P2Y receptors into the A and B subclasses has been recently proposed. Receptors in the A subclass (P2Y_{1,2,4,6,11}) are preferentially coupled to Gq or Gs proteins, thus mediating stimulation of phospholipase C and adenylyl cyclase. Receptors in the B subclass (P2Y_{12,13,14}) are instead mainly linked through G_i activation to inhibition of adenylyl cyclase activity (ibidem).

There are several similarities between the CysLT and the P2Y receptor classes. For example, the BLT1 receptor was originally reported as a potential nucleotide receptor based on homology with P2Y receptors but was subsequently found to be insensitive to nucleotides [27]. In a study with chimeric CysLT₁/P2Y₂ receptors, the chimera essentially behave as the CysLT₁ receptor and the activation was antagonized by a CysLT₁ receptor antagonist [28]. Crosstalk between purine and leukotriene systems has recently been reported, although the CysLT₁ receptor is not a P2Y nucleotide receptor [21]. P2Y₁ and CysLT receptors mediate co-release of purines and CysLTs in primary cultures of rat microglia [29].

Here we found that these leukotriene antagonists also inhibit the effects of nucleotides acting at P2Y receptors in dU937 cells, which are known to express a number of nucleotide receptors, such as P2Y₂ or P2Y₄ [30]. In an effort to characterize the P2 receptor subtypes subject to negative modulation by CysLT₁ receptor antagonists we studied the interactions of these compounds in human P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors stably expressed in 1321N1 astrocytoma cells. The latter have been chosen since these cells are devoid of endogenous P2Y receptors and for this reason have been widely utilized for transfecting and characterizing various P2Y receptor subtypes [31,32].

2. Materials and methods

2.1. Materials

1321N1 astrocytoma cells stably transfected with human P2Y_{1,2,4,6} receptors were kindly provided by Dr. Robert Nicholas (University of North Carolina, Chapel Hill, NC, USA). *myo*-[³H]Inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). Dowex AG 1-X8 resin was purchased from Bio-Rad (Hercules, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Life Technologies, Inc. (Rockville, MD, USA). Phosphate Buffer Saline (PBS), RPMI 1640, bovine serum albumin (BSA), EGTA, penicillin, streptomycin, L-glutamine, dimethyl sulfoxide (DMSO), probenecid, penicillamine, Hepes, UDP, UTP, ATP and hexokinase were from Sigma Chem. Co. (St. Louis, MO, USA). All salts for saline and Tris were from Merck (Darmstadt, Germany). ³H-LTD₄ (164–173 Ci/mmol) and Ultima Gold were purchased from Perkin-Elmer Life Sciences (Boston, MA, USA) and LTD₄ from Cayman Chemical Co. (Ann Arbor, MI, USA). Pranlukast and montelukast were a kind gift from Menarini (Menarini, Firenze, Italy). Fluo3/AM and pluronic F-127 were purchased from Molecular

Probes (Eugene, OR, USA). TRIZOL[®] Reagent, Superscript II RNA H⁻ Reverse Transcriptase and Platinum Taq DNA Polymerase were from Invitrogen (Milan, Italy). RQ1 RNase-free-DNase was purchased from Promega (Milan, Italy). Random hexamers were from Applied Biosystems (Milan, Italy). All other reagents were purchased from Sigma. Disposable culture flasks were from Corning Glassworks (Corning, NY, USA).

2.2. Cell culture and membrane preparation

1321N1 astrocytoma cells stably transfected with the human (h) P2Y₁, P2Y₂, P2Y₄, or P2Y₆ receptor were grown at 37 °C in a humidified incubator with 5% CO₂/95% air in Dulbecco's modified Eagle's medium (JRH Biosciences, Inc., Lenexa, KS, USA) and F12 (1:1) supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. The cells were grown to ~60% confluence for the experiments. For membrane preparation, human astrocytoma cells expressing the human P2Y₁ receptors were grown to approximately 80% confluence and then harvested. The cells were homogenized and suspended and centrifuged at 100 × g for 5 min at room temperature. The pellet was resuspended in 50 mM tris(hydroxymethyl) aminomethane (Tris)HCl buffer (pH 7.4). The suspension was homogenized with a polytron homogenizer (Brinkmann) for 10 s and was then recentrifuged at 20,000 × g for 20 min at 4 °C. The resultant pellets were resuspended in Tris buffer (pH 7.4), and the suspension was stored at -80 °C until the binding experiments. The protein concentration was measured with the Bradford assay [33]. Human promonocytic U937 cells (ATCC, Manassas, VA, USA) were routinely cultured in suspension in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C (5% CO₂) and differentiated for 96 h with 1.3% DMSO.

2.3. Total RNA isolation and RT-PCR analysis

Cells were collected by centrifugation. Total RNA was extracted from the cell pellet using the TRIZOL[®] Reagent according to manufacturer's instructions. PCR analysis was performed as previously described [34]. Briefly, after treatment of total RNA with RQ1 RNase-free-DNase, 1 µg of RNA was reverse-transcribed with Superscript II RNA H⁻ Reverse Transcriptase (200 U/sample) in the presence of 100 pmol of random hexamers. Aliquots (15% of the reverse-transcribed cDNA product) were amplified in each PCR assay with Platinum Taq DNA Polymerase (1.25 U/sample) in a 25 µl reaction mixture containing 20 pmol of 5' and 3' primers in a standard PCR buffer (50 mM KCl, 1.5 mM MgCl₂, 20 mM Tris-HCl, pH 8.4). For cDNA, control samples, which were not subjected to reverse transcription, were processed in parallel with the same experimental protocol to check for contamination of RNA with genomic DNA.

Amplifications were performed in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) for 40 cycles (typically 95 °C/45 s, 30 s at the annealing temperature ranging from 51 to 60 °C, depending on the specific receptor subtype; 72 °C/45 s) after an initially denaturation at 95 °C for 2 min. The following forward (Fw) and Reverse (Rw) oligonucleotide primers were used (size of PCR product):

P2Y ₁	Fw: 5'-CCTGCGAAGTTATTTTCATCTA-3'; Rw: 5'-GTTGAGACTTGCTAGACCTCT-3'
P2Y ₂	Fw: 5'-GCAGCATCCTCTTCCTCACCT-3'; Rw: 5'-CATGTTGATGGCGTTGAGGGT-3'
P2Y ₄	Fw: 5'-GGCATTGTCAGACACCTTGTA-3'; Rw: 5'-AAGGCACGAAGCAGACAGCAA-3'
P2Y ₆	Fw: 5'-CGCTTCCTCTTCTATGCCAA-3'; Rw: 5'-GTAGGCTGCTTGGTGATGTG-3'
P2Y ₁₁	Fw: 5'-ACTTCCTGTGCCCCATACTG-3'; Rw: 5'-GCTGTCCCCAGACACTTGAT-3'
P2Y ₁₂	Fw: 5'-CCCTCCAGAATCAACAGTTAT-3'; Rw: 5'-CGCTTTGCTTTAACGAGTTC-3'
P2Y ₁₃	Fw: 5'-TGTGTCGTTTTTCTTCGGTG-3'; Rw: 5'-TGCTGCCAAAAAGAGAGTTG-3'
P2Y ₁₄	Fw: 5'-CGCAACATATTCAGCATCGTGT-3'; Rw: 5'-GCTGTAATGAGCTTCGGTCTGAC-3'

2.4. Determination of inositol phosphates

The quantity of inositol phosphates was measured by a modification of the method of Gao et al. [35]. Agonists and antagonists were dissolved as stock solutions in PBS buffer (pH 7.4) and stored at -20°C . The hP2Y_{1,2,4,6}-1321N1 cells were grown to confluence in six-well plates in the presence of *myo*-[³H]inositol (2 $\mu\text{Ci/ml}$) for 24 h. After an additional incubation of 20 min with 20 mM LiCl at room temperature, cells were treated with agonist or antagonist. Agonists used were as follows: hP2Y₁, 2-MeSADP; hP2Y₂, UTP; hP2Y₄, UTP; hP2Y₆, UDP. The reaction was terminated upon aspiration of the medium and addition of cold formic acid (20 mM). After 30 min, supernatants were neutralized with NH₄OH, and applied to Bio-Rad Dowex AG 1-X8 anion exchange columns. All of the columns were washed with water followed by a 60 mM sodium formate solution containing 5 mM sodium tetraborate. Total inositol phosphates were eluted with 1 M ammonium formate containing 0.1 M formic acid, and radioactivity was measured using a liquid scintillation counter.

2.5. Radioligand binding assays

Equilibrium binding studies in intact dU937 cells were performed as previously described [36]. Briefly, cells were incubated at 25°C for 60 minutes using 0.3 nM ³H-LTD4 and unlabeled compounds at the indicated concentrations. dU937 cells ($(5-10) \times 10^6$ cells/sample), saline solution pH 7.4, 20 mM CaCl₂ and 20 mM penicillamine were added to the incubation mixture to achieve a final volume of 500 μl . Unbound ligand was separated by centrifugation; the pellet was washed once with 1 ml of saline solution at 4°C and dissolved in 1N NaOH and radioactivity measured in a liquid scintillation counter.

P2Y₁ receptor binding experiments were performed as previously described [35,37]. Briefly, membranes (40 μg protein) from astrocytoma cells stably expressing human P2Y₁ receptors were incubated with [³H]MRS2279 (8 nM) for 30 min at 4°C in a total assay volume of 200 μl . The radiolabeled ligand concentration used in all assays approximated the K_d value of 8 nM at the P2Y₁ receptor. Binding reactions were terminated by filtration through Whatman GF/B glass-fiber filters under reduced pressure with a MT-24 cell harvester (Brandel, Gaithersburg, MD, USA), and radioactivity was determined with a 1414 liquid scintillation counter (Wallac, Win Spectral, Perkin-Elmer Life Sciences, Downers Grove, IL, USA). Data were expressed as mean \pm S.E.

2.6. Calcium mobilization assay

Determination of cytosolic free Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) in dU937 cells was performed as previously described [36]. Briefly, dU937 cells were incubated for 30 min at 30 °C in the dark with 2 μM Fluo3/AM. After loading, Fluo3/AM was removed and cells were further incubated for 30 min at 30 °C to complete the hydrolysis of the fluorescent indicator. When indicated hexokinase was added to UDP stock solution (50 Units/ml) and cells (1 Unit/ml) accordingly to Kumari et al. [38]. Cells were then centrifuged, diluted to the concentration of 10^6 cells/ml, transferred to the spectrofluorimeter (Perkin-Elmer LS50) and fluorescence was monitored at 37 °C (506 nm excitation, 530 nm emission). Calibration was performed by adding 2 μM ionomycin and 100 μM digitonin (F_{max}) and by adding 5 mM EGTA and 60 mM Tris-base (F_{min}). $[\text{Ca}^{2+}]_i$ was calculated according to Tsien et al. [39] with a $K_d = 864$ [40]. $[\text{Ca}^{2+}]_i$ elevation was expressed as stimulated over basal (S/B).

Human astrocytoma cells stably expressing human P2Y receptors were cultured in Dulbecco's modified Eagle's medium (DMEM, JRH Biosciences, Inc., Lenexa, KS, USA) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units penicillin/ml, 100 μg streptomycin/ml, 2 μmol glutamine/ml, and 500 μg geneticin/ml. For the assay mobilization, cells were grown overnight in 100 μl of media in 96 well flat bottom plates at 37 °C at 5% CO_2 or until approx. 60–80% confluence. The calcium assay kit (Molecular Devices, Sunnyvale, CA, USA) was used as directed with no washing of cells, and with probenecid added to the loading dye at a final concentration of 2.5 mM to increase dye retention. Cells were loaded upon addition of 50 μl of dye containing probenecid to each well and incubated for 45 min at room temperature prior to addition of agonist. The compound plate was prepared using dilutions of various compounds in Hanks Buffer with 20 mM HEPES, pH 7.2. For antagonist studies. The antagonist was added 20 mM prior to the addition of agonist to the sample plate. Samples were performed in duplicate using a Molecular Devices FlexStation I at room temperature. Cell fluorescence (excitation = 485 nm, emission = 525 nm) was monitored following exposure to compound. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure.

2.7. Statistical analysis

Concentration response-curves were analyzed and parameters calculated using GraphPad Prism software (GraphPad, San Diego, CA, USA). Statistical comparison among different parameters has been performed with GraphPad software utilizing the extra sum of square principle. Data were expressed as mean \pm S.E.M.

3. Results

3.1. P2Y receptor expression profile in dU937 cells

The expression of P2Y receptors in this differentiated macrophage cell line was demonstrated. RT-PCR analysis performed in dU937 cells with primers specifically designed for the various cloned P2Y receptors (see Section 2) revealed bands corresponding to the expected amplification products of P2Y₂ (502 bp), P2Y₄ (551 bp), P2Y₆ (480 bp), P2Y₁₁ (499 bp), P2Y₁₃ (578 bp) and P2Y₁₄ (102 bp) receptors (Fig. 1). These primers did not

generate additional bands. No signal was detected in parallel RNA samples that did not undergo retro-transcription demonstrating that the amplified product was not due to contamination of RNA with genomic DNA (data not shown). No amplification products were observed with primers specific for P2Y₁ (318 bp) and P2Y₁₂ (1127 bp) receptors, suggesting that these receptors are not expressed in dU937 cells. In a positive control experiment using the same PCR primers for these two P2Y subtypes, amplified products with the expected MW were obtained using cDNA from human brain (data not shown).

3.2. Characterization of nucleotide-mediated [Ca²⁺]_i transients in dU937 cells

Consistent with the presence of P2Y receptors in the dU937 cells, extracellular nucleotides were found to elicit functional effects at micromolar concentrations. Fig. 2A–C shows that the indicated nucleotides were able to trigger a concentration-dependent increase in [Ca²⁺]_i with an EC₅₀ value of 3.9 ± 0.4 μM reaching a maximum at approximately 100 μM for ATP, EC₅₀ = 64 ± 5 μM, reaching a maximum at approximately 1 mM for UDP and EC₅₀ = 7.1 ± 1.2 μM, reaching a maximum at approximately 100 μM for UTP. The maximal response was 5.2-fold stimulated over basal for ATP, 4.7-fold for UDP and 3.9-fold for UTP. Furthermore, treatment with hexokinase of both UDP stock solution (50 U/ml) and dU937 cells (1 U/ml), to exclude UTP contamination or extracellular conversion of UDP, did not have any effect on UDP potency.

3.3. Effect of montelukast and pranlukast on nucleotide-mediated [Ca²⁺]_i transients in dU937 cells

The functional effects of exposure of the dU937 cells to extracellular nucleotides were antagonized by CysLT₁ receptor antagonists. Fig. 3 shows that 5 min pretreatment with montelukast or pranlukast were found to inhibit [Ca²⁺]_i transients elicited by UDP and UTP with IC₅₀ values of 4.3 ± 0.3 and 7.7 ± 2.7 μM, respectively, for montelukast (Fig. 3A and B), and with IC₅₀ values of 1.6 ± 0.4 and 4.5 ± 0.8 μM respectively, for pranlukast (Fig. 3C and D).

Furthermore, both montelukast and pranlukast have been found to affect the maximal efficacy of UDP-induced [Ca²⁺]_i transients, thus lowering the upper plateau of the concentration–response curves (Fig. 4A and B, *p* < 0.05), clearly deviating from apparent simple competition, i.e. competitive surmountable antagonism. In addition, pranlukast also affected the apparent UDP potency (Fig. 4B, *p* < 0.01). These data, therefore, are consistent either with a competitive insurmountable antagonism, characteristic of irreversible compounds, or with a noncompetitive antagonism, characteristic of allosteric compounds, for both antagonists [41]. Similar results were obtained with UTP-induced [Ca²⁺]_i transients (data not shown). In addition, Fig. 4C shows a typical trace of UDP-induced [Ca²⁺]_i before and after treatment with 6 μM montelukast. Treatment with montelukast alone did not modify [Ca²⁺]_i (data not shown).

3.4. Effect of nucleotides on [³H]LTD₄ binding to the CysLT₁ receptor

In order to establish whether or not UTP and UDP were indeed able to compete for binding at the LTD₄ orthosteric binding site, we performed equilibrium binding studies in intact dU937 cells using [³H]LTD₄ as a labeled ligand (Fig. 5). As expected, binding was

significantly inhibited by LTD₄ in a concentration-dependent manner, but neither nucleotide was able to compete with [³H]LTD₄ binding sites.

3.5. Effect of montelukast and pranlukast on signaling and receptor binding in astrocytoma cells

Inhibition of nucleotide-induced production of inositol phosphates and calcium transients by montelukast and pranlukast was studied in 1321N1 human astrocytes expressing P2Y_{1,2,4,6} receptors. Effects on signaling at P2Y₁₁, P2Y₁₃ and P2Y₁₄ receptors, although present in the dU937 cells, were not investigated. P2Y₁ receptors were included, although they are not expressed in the dU937 cells, because this is one of two P2Y receptor subtypes at which radioligand binding characterization is feasible.

Fig. 6A indicated that montelukast affected the concentration–response curves for intracellular Ca²⁺ changes induced by 2-MeSADP acting at the P2Y₁ receptors expressed in 1321N1 astrocytoma cells. In agreement with data obtained in dU937 cells, montelukast does not behave as a competitive, surmountable antagonist. Full concentration–response curves for the inhibition of the effects of 2-MeSADP at P2Y₁ receptors were measured for montelukast and pranlukast (Fig. 6B). Results indicated that the production of inositol phosphates in human P2Y₁ receptor-expressing 1321N1 astrocytoma cells in response to 30 nM 2-MeSADP was inhibited in a concentration-dependent manner with IC₅₀ values of 0.122 ± 0.037 and 0.028 ± 0.013 μM, for montelukast and pranlukast, respectively.

Since the leukotriene antagonists displayed functional antagonism of P2Y₁ receptor-elicited effects, we examined the ability to interfere with radioligand binding at that subtype. The specific P2Y₁ receptor antagonist [³H]MRS2279 was used as a high affinity radioligand in binding to membranes from astrocytoma cells stably expressing human P2Y₁ receptors [37]. This binding of this radioligand is competitive with other nucleotide ligands of the P2Y₁ receptor, both agonists and antagonists. Specific binding of [³H]MRS2279 was defined in these experiments using the antagonist MRS2179 (10 μM). Essentially no inhibition of binding of [³H]MRS2279 was detected in the presence of 10 μM montelukast and pranlukast (Fig. 6C), both of which antagonized the P2Y₁ receptor-mediated effects of 2-MeSADP. Binding was displaced by the P2Y₁ antagonist MRS2179, as expected (not shown).

CysLT receptors have been detected in astrocytes [45], and PCR analysis of 1321N1 astrocytoma cells indicated a low level of expression of both CysLT₁ and CysLT₂ receptors (data not shown). Nevertheless, attempts to detect [³H]LTD₄ binding in membranes from 1321N1 astrocytoma cells stably expressing human P2Y₁ receptors indicated only non-specific binding levels (data not shown).

Montelukast inhibited the functional effects of UDP acting as agonist at hP2Y₆ receptors (Fig. 7). In good agreement with data obtained in dU937 cells, once again montelukast behaved as an insurmountable antagonist. The production of inositol phosphates in human P2Y₆ receptor-expressing 1321N1 astrocytoma cells in response to 300 nM UDP was inhibited in a concentration-dependent manner with IC₅₀ values of 0.859 ± 0.053 and 0.150 ± 0.065 μM, for montelukast and pranlukast, respectively. Less pronounced effects of the CysLT₁ antagonists were seen on the functional responses to UTP acting at P2Y₂ and P2Y₄

receptors. Montelukast (10 μM) also blocked the human P2Y₂ receptor signaling through PLC induced by the agonist UTP (Fig. 8A), while pranlukast (10 μM) had no significant effects on UTP-induced human P2Y₂ receptor activation (data not shown). Montelukast (10 μM) also had a slight effect on the human P2Y₄ receptor signaling (activation of PLC, Fig. 8B), which was more evident in the assay of $[\text{Ca}^{2+}]_i$ mobilization (Fig. 8C). Control 1321N1 astrocytoma cells did not have a significant response of either PLC or $[\text{Ca}^{2+}]_i$ mobilization when exposed to ADP, ATP, UDP, or UTP (data not shown). Control experiments using carbachol to stimulate $[\text{Ca}^{2+}]_i$ mobilization via an endogenous m3 muscarinic acetylcholine receptor in the control astrocytoma cells demonstrated a lack of significant inhibition by montelukast (Fig. 8D).

4. Discussion

A relationship between UDP and the CysLT₁ receptor has already been suggested, with the hypothesis that UDP may act directly on the receptor [42,43].

Nevertheless, the concentrations of CysLT₁ antagonists required to functionally antagonize the effects of UDP in this study are not comparable to the nM concentrations at which the compounds act at the CysLT₁ receptor. Although it does not establish the molecular basis for interaction, this study expands the range of possible interactions to other uracil and adenine nucleotides.

In this study, montelukast and pranlukast were found to inhibit nucleotide-induced calcium mobilization in a human monocyte-macrophage like cell line, DMSO-differentiated U937 (dU937) in an insurmountable manner. dU937 cells were shown to express P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₃ and P2Y₁₄ receptors. Therefore, these antagonists were studied functionally in a heterologous expression system for the human P2Y receptors. In 1321N1 astrocytoma cells stably expressing hP2Y_{1,2,4,6} receptors, CysLT₁ antagonists inhibited both the P2Y agonist-induced activation of phospholipase C and intracellular Ca^{2+} mobilization. The inhibition was concentration-dependent but in an insurmountable manner. In control astrocytoma cells expressing an endogenous M3 muscarinic receptor, 10 μM montelukast had no effect on the carbachol-induced rise in intracellular Ca^{2+} . Thus, these data demonstrated that CysLT₁ receptor antagonists interact functionally with signaling pathways of P2Y receptors, and independent of another PLC-coupled receptor.

Furthermore, ligand binding studies using [³H]LTD₄ excluded the possibility of competitive nucleotide binding to the CysLT₁ receptor in dU937 cells, but not eliminating the possibility of an allosteric interaction at the CysLT₁ receptor level. Another possible explanation for these effects is that the leukotriene antagonists are interacting directly with the P2Y receptors, to which they are related in the phylogenetic dendrogram of GPCRs [15]. However, in the P2Y₁ receptor-expressing astrocytoma cells the binding of a specific antagonist (nucleotide) radioligand of the P2Y₁ receptor, [³H]MRS2279, was not displaced by the leukotriene antagonists. Since MRS2279 is a competitive ligand, if the interaction of the leukotriene antagonist is directly with this receptor, it would necessarily be through an allosteric interaction as already suggested by data obtained in dU937 cells (see Figs. 4 and 5). Other examples of allosteric modulation of P2Y receptors have been reported [35].

Another possibility is that the antagonism occurs at a yet unidentified signaling step subsequent to activation of P2Y, but not M3 muscarinic, receptors. Thus, the functional inhibition of the nucleotide-elicited effects was either through direct allosteric interaction with the receptor or through a signaling pathway common to the P2Y receptors but not characteristic of cholinergic signaling.

Furthermore, we have recently shown that activation of P2Y receptors with extracellular nucleotides induced heterologous desensitization of the CysLT₁ receptor in dU937 cells, but not receptor trafficking or internalization. Conversely, LTD₄-induced CysLT₁ receptor activation had no effect on P2Y receptor responses, suggesting that the latter have a hierarchy in producing desensitizing signals [14]. Thus, while a putative heterodimerization between CysLT₁ and P2Y receptors might as well explain our results, it is unlikely to occur in our system, considering the lack of cross-desensitization, the different mechanism of agonist- or nucleotides-induced CysLT₁ desensitization, and the different trafficking induced by LTD₄ or by extracellular nucleotides signals [14].

In conclusion, our data demonstrated that montelukast and pranlukast non-competitively blocked P2Y signaling in several cell systems, but in a relatively nonsubtype-specific manner. The functional antagonism was especially evident at heterologously expressed P2Y₁ and P2Y₆ receptors, at which IC₅₀ values for inhibition of calcium mobilization were <1 μM. Since the inhibition of P2Y₁ receptor-elicited effects occurred without affecting specific nucleotide binding, if there is a direct interaction, it may be allosteric. The implications of these data for the clinical use of leukotriene antagonists is yet to be explored, with the question if some of their effects at therapeutic doses [44] may be related to their ability to inhibit the signaling pathways of P2Y receptors for extracellular nucleotides. These observations should foster the study of possible implications for the clinical use of these compounds in asthma or in other inflammatory conditions.

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Abbreviations

DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
FBS	fetal bovine serum
IP₃	inositol trisphosphate
CysLT	cysteinyl leukotriene
GPCR	G protein-coupled receptor

MRS2179	<i>N</i> ⁶ -methyl-2'-deoxyadenosine-3',5'-bisphosphate
MRS2279	(1' <i>R</i> ,2' <i>S</i> ,4' <i>S</i> ,5' <i>S</i>)-4-(2-chloro-6-methylamino-purin-9-yl)-1-[(phosphato)-methyl]-2-(phosphato)-bicyclo[3.1.0]hexane
PLC	phospholipase C

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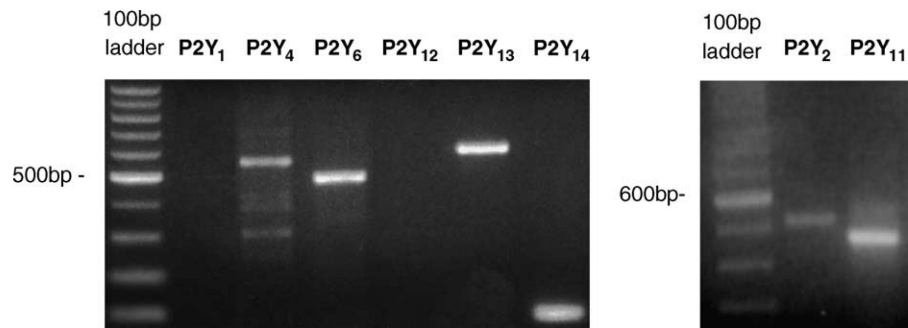


Fig. 1.

Expression of P2Y_{2,4,6,11,13,14} in dU937 cells. Total RNA was subjected to RT and the resulting cDNA was amplified with primers specifically designed for human P2Y_{1,2,4,6,11,12,13,14} receptors. PCR products were separated on 2% ethidium bromide-stained agarose gel and visualized under UV light. Identification of the PCR products was made on the basis of their predicted sizes (see Section 3).

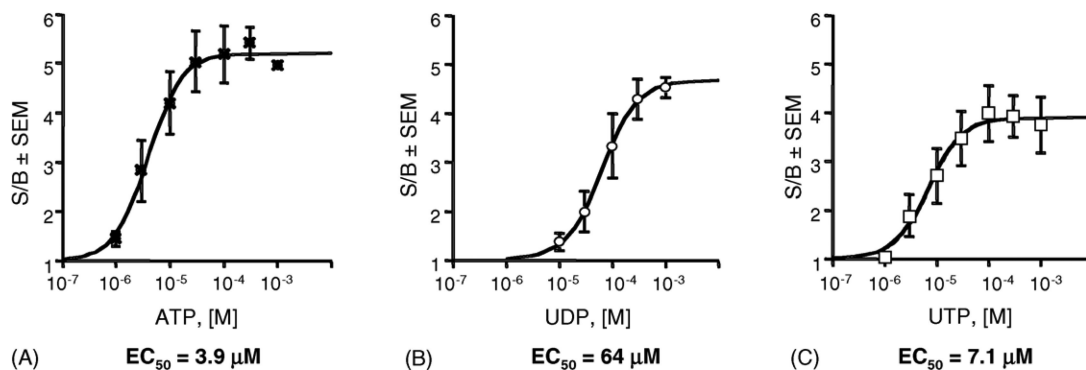


Fig. 2.

Concentration–response curves of the $[Ca^{2+}]_i$ transient induced by nucleotides in dU937 cells. Fluo3/AM loaded cells were challenged with increasing concentrations of ATP (A), UDP (B), and UTP (C). When hexokinase was present in the UDP experiment, the response curve was unchanged (EC_{50} 69 μ M), indicating that this effect was not the result of contamination by UTP. Results are expressed as the ratio of stimulated over basal (S/B) \pm S.E.M. and represent the mean from three independent experiments analyzed simultaneously.

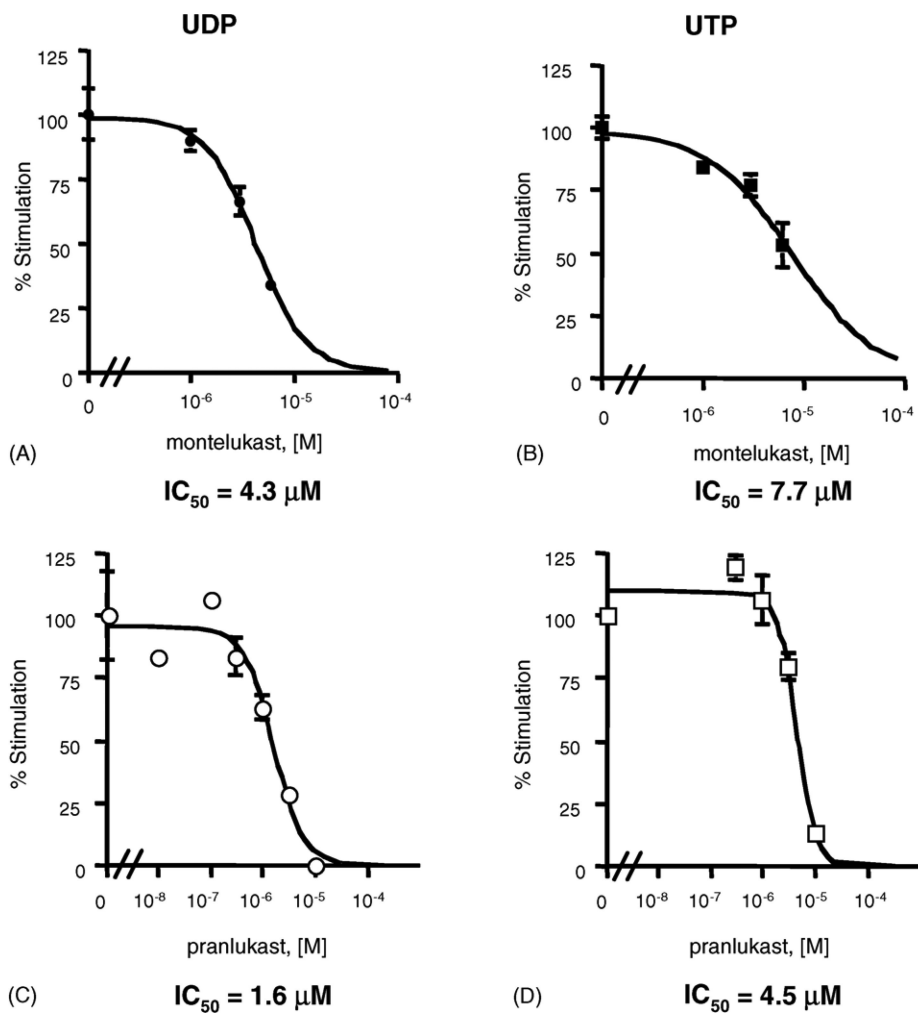
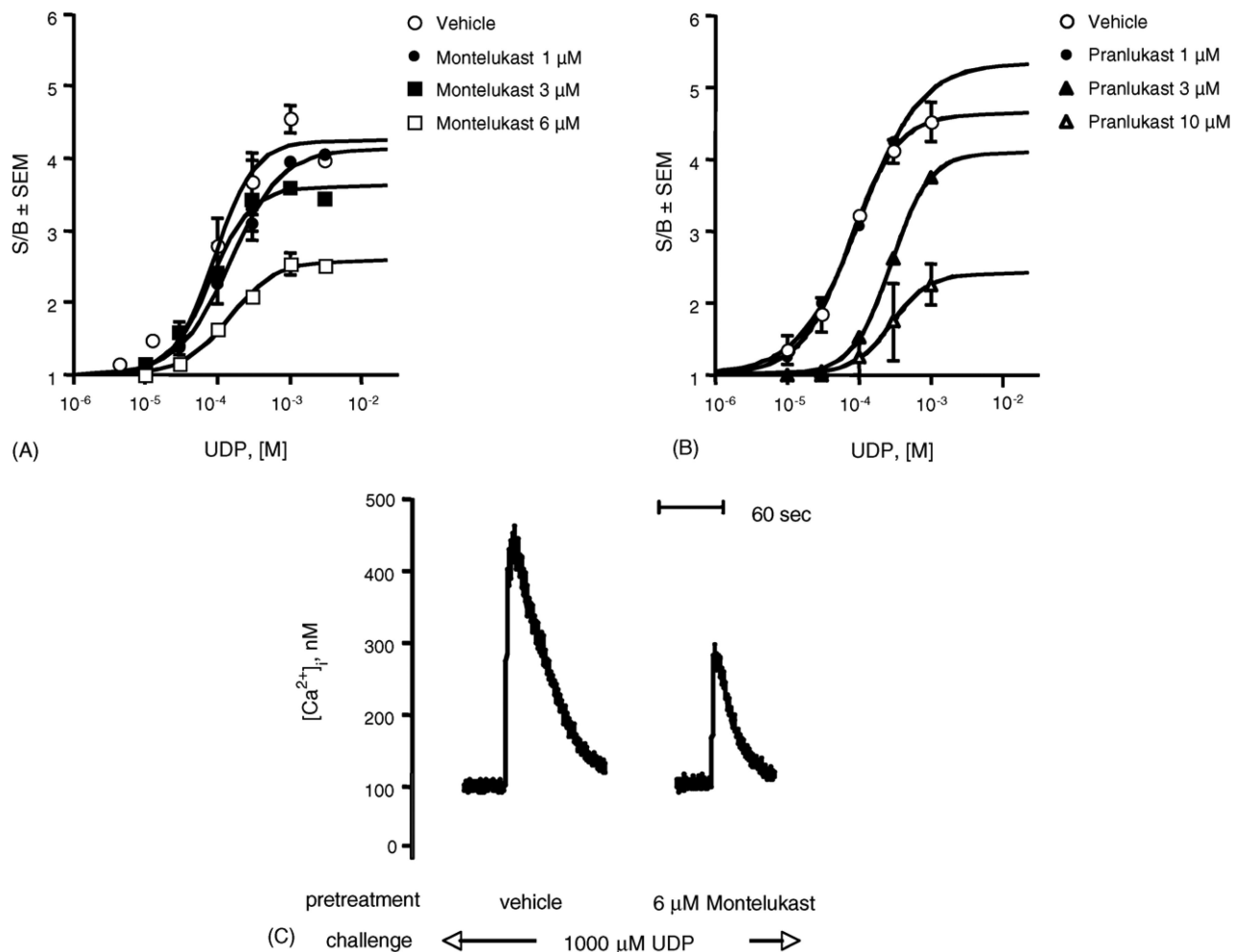


Fig. 3. Effect of pranlukast and montelukast on nucleotide-induced $[Ca^{2+}]_i$ transients in dU937 cells. Fluo3/AM loaded cells were challenged with $300 \mu M$ UDP (A and C) and $30 \mu M$ UTP (B and D) in the presence of increasing concentrations of montelukast and pranlukast (5 min pretreatment). Results are expressed as the ratio of stimulated over basal (S/B) \pm S.E.M. and represent the mean from at least three independent experiments analyzed simultaneously.

**Fig. 4.**

Effect of pranlukast and montelukast on the concentration–response curve for the $[Ca^{2+}]_i$ transients induced by UDP in dU937 cells. Fluo3/AM loaded cells were challenged with increasing concentrations of UDP in the absence and presence of the indicated concentrations of montelukast (A) and pranlukast (B) (5 min pretreatment). Results are expressed as the ratio of stimulated over basal (S/B) \pm S.E.M. and represent the mean from two independent experiments analyzed simultaneously. (C) Typical trace of UDP-induced $[Ca^{2+}]_i$ transients before and after treatment with 6 μ M montelukast.

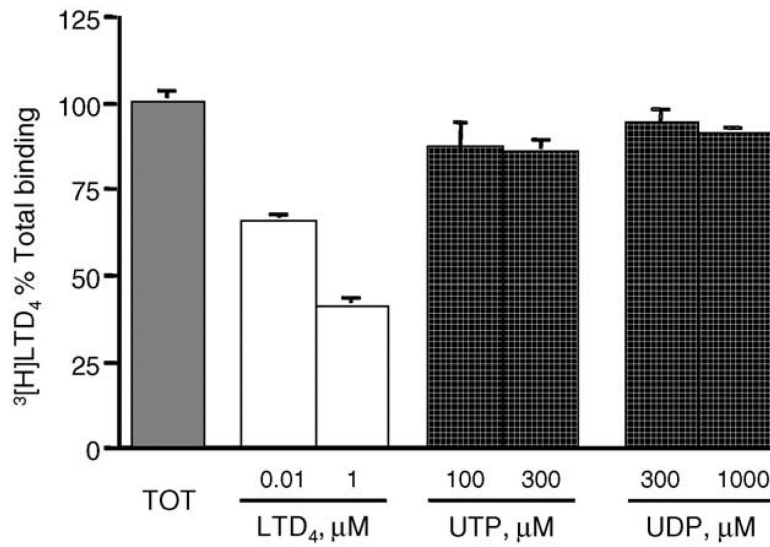


Fig. 5. Equilibrium binding of [³H]LTD₄ in intact dU937 cells. Data are presented as percent total [³H]LTD₄ binding displaced by the indicated concentrations of unlabeled LTD₄, UTP or UDP. Data shown are mean ± S.E.M. from two independent experiments performed in triplicates.

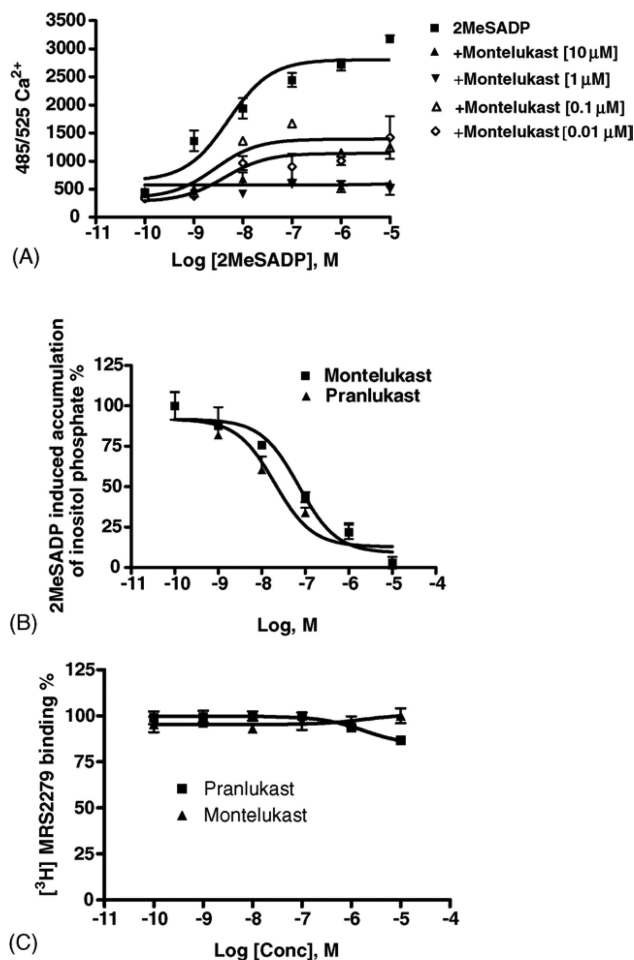


Fig. 6. Functional effects of leukotriene antagonists on 2-MeSADP-induced signaling in stably transfected astrocytoma cells. (A) Effect of montelukast on concentration–response curves for intracellular Ca^{2+} changes induced by 2-MeSADP acting at $P2Y_1$ receptors expressed in 1321N1 astrocytoma cells. The cells were pre-treated for 20 min at room temperature with antagonist before application of the agonist 2-MeSADP. The fluorescence emission at 525 nm with excitation at 485 nm is proportional to $[Ca^{2+}]_i$. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure. (B) Concentration-dependence of inhibition by montelukast and pranlukast of inositol phosphate production induced by 30 nM 2-MeSADP in human $P2Y_1$ transfected 1321N1 human astrocytoma cells. (C) Effect of montelukast and pranlukast on the binding of [^3H]MRS2279 to membranes from astrocytoma cells stably expressing the human $P2Y_1$ receptor. Data shown are mean \pm S.E.M. from three independent experiments performed in duplicate.

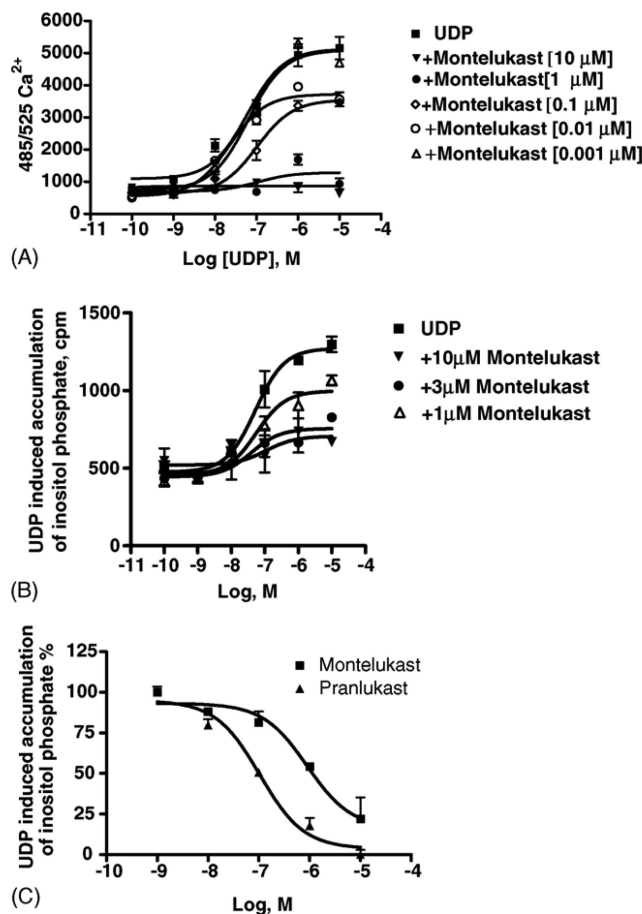


Fig. 7. Functional effects of leukotriene antagonists on UDP-induced signaling in stably transfected P2Y₆ receptor-expressing astrocytoma cells. (A) Effect of montelukast on concentration-response curves for intracellular Ca²⁺ changes induced by UDP acting at P2Y₆ receptors expressed in 1321N1 astrocytoma cells. The cells were pre-treated for 20 min at room temperature with antagonist before application of the agonist UDP. The fluorescence emission at 525 nm with excitation at 485 nm is proportional to [Ca²⁺]_i. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure. Data shown are mean ± S.E.M. from three independent experiments performed in duplicate. (B) Inositol phosphate production in human P2Y₆ transfected 1321N1 human astrocytes. After labeling with *myo*-[³H]inositol (1 μCi/10⁶ cells) for 24 h, the cells were treated for 20 min at 37 °C with montelukast (10, 3 and 1 μM concentration) in the presence of LiCl, followed by addition of the agonist, UDP, for another 30 min. The quantity of inositol phosphates was analyzed after the extraction through Dowex AG 1-X8 columns (see Section 2). Data shown are mean ± S.E.M. from two independent experiments performed in duplicate. (C) Concentration dependence of inhibition by montelukast and pranlukast of inositol phosphate production induced by 300 nM UDP in human P2Y₆ transfected 1321N1 human astrocytes. Data shown are mean ± S.E.M. from three independent experiments performed in duplicate.

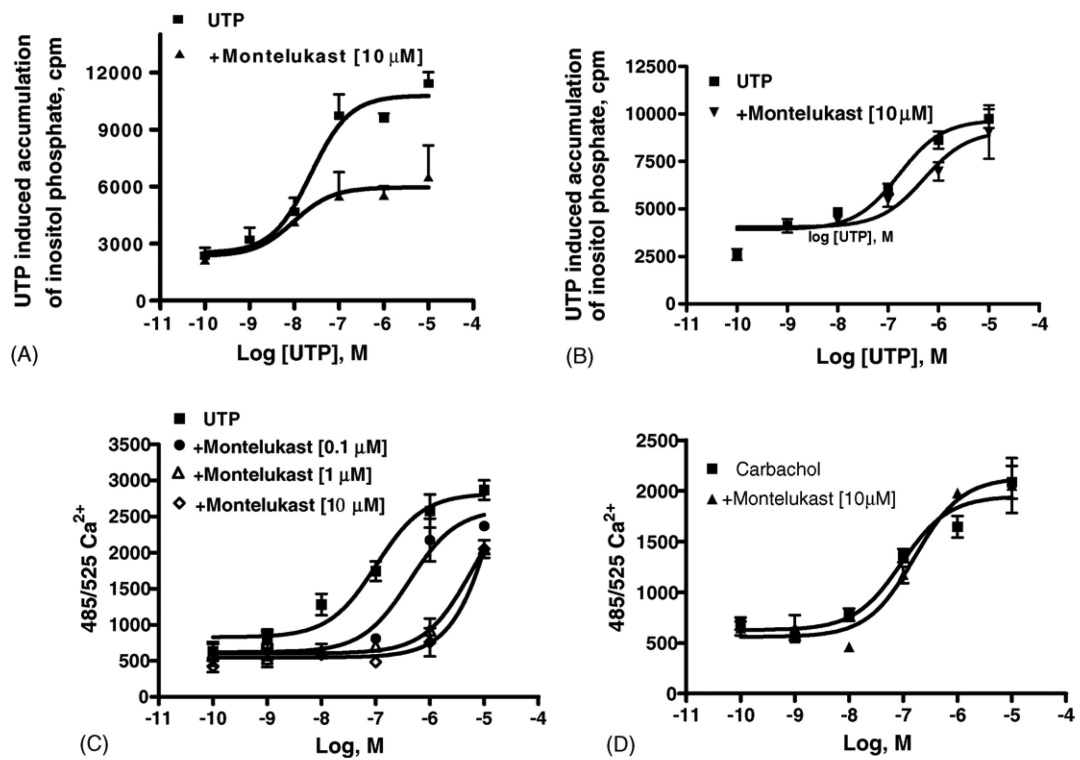


Fig. 8.

Functional effects of leukotriene antagonists on signaling in astrocytoma cells (either in control cells or in cells stably expressing P2Y receptors). (A) Inositol phosphate production in human P2Y₂-receptor transfected 1321N1 human astrocytes. After labeling with *myo*-[³H]inositol (1 μCi/10⁶ cells) for 24 h, the cells were treated for 30 min at 37 °C with montelukast (10 μM concentration) in the presence of LiCl, followed by addition of the agonist, UTP, for another 30 min. The quantity of inositol phosphates was analyzed after the extraction through Dowex AG 1-X8 columns (see Section 2). Data shown are mean ± S.E.M. from three independent experiments performed in duplicate. (B) Inositol phosphate production induced by UTP in human P2Y₄-receptor transfected 1321N1 human astrocytes. (C) Effect of montelukast on concentration–response curves for intracellular Ca²⁺ changes induced by UTP acting at P2Y₄ receptors stably expressed in 1321N1 astrocytoma cells. The fluorescence emission at 525 nm with excitation at 485 nm is proportional to [Ca²⁺]_i. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure. (D) Lack of effect of montelukast on concentration–response curves for intracellular Ca²⁺ changes induced by carbachol acting at endogenous M3 muscarinic acetylcholine receptors in control 1321N1 astrocytoma cells. The cells were pre-treated for 20 min at room temperature with antagonist before application of the agonist carbachol. Data shown are mean ± S.E.M. from two independent experiments performed in triplicate.