ACCELERATED COMMUNICATION

Molecular Cloning and Characterization of a Second Human Cysteinyl Leukotriene Receptor: Discovery of a Subtype Selective Agonist

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ABSTRACT

The cysteinyl leukotrienes (CysLTs) are potent biological mediators in the pathophysiology of inflammatory diseases, in particular of airway obstruction in asthma. Pharmacological studies have suggested the existence of at least two types of CysLT receptors, designated CysLT₁ and CysLT₂. The CysLT₁ receptor has been cloned recently. Here we report the molecular cloning, expression, localization, and functional characterization of a human G protein-coupled receptor that has the expected characteristics of a CysLT₂ receptor. This new receptor is selectively activated by nanomolar concentrations of CysLTs with a rank order potency of LTE₄ > LTD₄ > LTC₄. The leukotriene analog BAY u9773, reported to be a dual CysLT₁/CysLT₂ antagonist, was found to be an antagonist at CysLT₁ sites but acted as a partial agonist at this new receptor. The structurally different CysLT₁ receptor-selective antagonists zafirlukast, montelukast, and MK-571 did not inhibit the agonist-mediated calcium mobilization of CysLT₂ receptors at physiological concentrations. Localization studies indicate highest expression of CysLT₂ receptors in adrenal glands, heart, and placenta; moderate levels in spleen, peripheral blood leukocytes, and lymph nodes; and low levels in the central nervous system and pituitary. The human CysLT₂ receptor gene is located on chromosome 13q14.12–21.1. The new receptor exhibits all characteristics of the thus far poorly defined CysLT₂ receptor. Moreover, we have identified BAY u9773 as a CysLT₂ selective agonist, which could prove to be of immediate use in understanding the functional roles of the CysLT₂ receptor.

Cysteinyl leukotrienes (CysLTs) are the products of the 5-lipoxygenase pathway in arachidonic acid metabolism. They are predominantly produced by myeloid cells associated with the inflammatory responses (Samuelsson et al., 1987) and are potent constrictors of pulmonary smooth muscles (Dahlén et al., 1980), trachea, and parenchyma in human airways, where they induce microvascular permeability (Dahlén et al., 1981) and mucus secretion (Marom et al., 1982). Leukotrienes have been implicated in a number of pathological inflammatory diseases including asthma, allergic rhinitis, inflammatory bowel disease, and psoriasis (Busse and Gaddy, 1991). The effects of CysLTs are mediated via specific plasma membrane receptors belonging to the superfamily of G protein-coupled receptors. There is evidence for the existence of two CysLT receptor subtypes (Fleisch et al., 1982; Labat et al., 1992; Coleman et al., 1995; Metters, 1995): CysLT₁ and the CysLT₂ receptors, the latter of which encompasses all receptors that cannot be inhibited by CysLT₁-specific antagonists (Coleman et al., 1995). The CysLT₁ receptor has been studied intensively because of the availability of CysLT₁-specific antagonists (Saussy et al., 1999). Recently, a cDNA encoding a CysLT₁ receptor was cloned (Lynch et al., 1999; Sarau et al., 1999). The pharmacological profile of the cloned CysLT₁ showed a rank order potency of LTD₄ > LTC₄ > LTE₄ and was potently inhibited by the CysLT₁ antagonists pranlukast, montelukast, zafirlukast, and pobilukast. The CysLT₂ receptor, on the other hand, is pharmacologically less defined, mainly be-

ABBREVIATIONS: CysLT, cysteinyl leukotrienes; PCR, polymerase chain reaction; GPCR, G protein-coupled receptor; HEK 293T, human embryonic kidney cells stably expressing the simian virus 40 large T antigen.
cause of the lack of selective agonists and antagonists. In man, CysLT₂ receptors have been indirectly shown to be responsible for contracting pulmonary veins, contractions that were resistant to a number of CysLT₁-selective antagonists (Labat et al., 1992).

In our quest to identify the natural ligands of orphan

Fig. 1. Nucleotide- and deduced amino acid sequence of the human HPN321(CysLT₂) receptor as derived from clone RP11–108P5 (AL137118). Putative poly-adenylation signals are underlined. Position 2123 to 2430 corresponds to an expressed sequence tag (AW235714) found in the dBEST database from a subtracted kidney library. The sequence of HPN321 has been deposited in GenBank (accession no. AF279611).
GPCRs, we cloned in silico a receptor, HPN321, that exhibited moderate similarity to the CysLT1 receptor. We describe here the pharmacological characterization of this novel receptor and conclude that we cloned a receptor exhibiting the expected characteristics of the CysLT2 receptor.

**Experimental Procedures**

**Materials.** LTB₄, LTC₄, LTD₄, and LTE₄ were from Cayman Chemical (Ann Arbor, MI). BAY u9773 and MK-571 were from BIOMOL Research Laboratories (Plymouth Meeting, PA). LY-17883 was from Sigma (St. Louis, MO). Zafirlukast (ICI 204,219; Accolate) and montelukast (MK-476; Singular) were purchased from the local pharmacy. All other standard chemicals used were either from Fisher or Sigma.

**Identification, Cloning, and Sequencing of HPN321 and CysLT₁ receptors.** A human chromosomal clone (AL137118) was identified during our systematic computational queries of novel DNA sequences coding for GPCR-related proteins. The corresponding open reading frame was identified using TBLAST algorithm and known GPCR protein sequences as queries. The DNA sequence was used to design two sets of nested primers (first set 5'-GATAGATTGGCCTCCGTGTTACATT-3' [−112 to −89] and 5'-GAAGATGGACACAGGGATACAAGG-3' [1064 to 1087] and second set 5'-ATGTAACTCAGTAGCGAAAGGGA-3' [−75 to −53] and 5'-ACAGGTCTCTCATCTAAGAGCTCTTT-3' [1063 to 1040]). The first polymerase chain reaction (PCR) was carried out with the ExpandLong Template PCR system (Hoffman La Roche, Nutley, NJ) on 0.1 g of human genomic DNA (CLONTECH, Palo Alto, CA) according to the manufacturer’s recommendation. Second PCR was performed with 2 µl of first-round PCR product and 1.0 U of Taq polymerase (Promega, Madison, WI) in 50 µl of the buffer supplied by the manufacturer containing 2.5 mM MgCl₂ and was carried out for 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. The resulting product was analyzed by agarose gel electrophoresis and subcloned into pCDNA3.1 (Invitrogen, San Diego, CA). The accuracy of the sequence was confirmed by DNA sequencing using a dyeoxy termination kit from Amersham Pharmacia Biotech (Piscataway, NJ). CysLT₁ was cloned from an expressed sequence tag available through the I.M.A.G.E. consortium [1064 to 1087] and second set 5'-ATGTAATCAGTATACCTGGG-9'.

**Expression in HEK 293T Cells.** HPN321/CysLT₁ receptor plasmids were transiently expressed in human embryonic kidney cells stably expressing the simian virus 40 large T antigen (HEK 293T) cells using LipofectAMINE PLUS (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Transfections were done in 100-mm tissue culture dishes and seeded after 24 h into microtiter plates for subsequent assays. Assays (see below) were performed 48 h after cell transfections. Cell lines stably expressing HPN321 were established by selecting hygromycin-resistant clones.

**Measurement of Agonist-Induced Calcium Release.** Calcium mobilization assays were carried out using transiently transfected HEK 293T-HPN321 or CysLT₁ cells loaded with Fluo-4 AM fluorescent indicator dye (Molecular Probes, Eugene, OR) in the fluorescent imaging plate reader system (Molecular Devices, Sunnyvale, CA). Briefly, cells were seeded in poly-D-lysine-treated black microtiter plates at 8 × 10⁶ cells/well and grown to confluence. The cells were loaded with 2 µM Fluo-4 in growth medium (Dulbecco's modified Eagle's medium, 10% fetal bovine serum) supplemented with 2.5 mM probenecid for 1 h at 37°C, 5% CO₂. The cells were washed thrice with Hanks' balanced salt solution containing 10 mM HEPES and 2.5 mM probenecid. Calcium transient curves were generated by reading baseline fluorescence values for 10 s, followed by addition of test compounds. For the first minute, fluorescence values were collected in 1-s intervals; for the next 2 min, data were collected in 3-s intervals. For calculation of dose-response curves, the peak fluorescence values for each agonist concentration were determined and analyzed by nonlinear regression using PRISM software (GraphPad, San Diego, CA). Antagonist studies were performed under the same conditions but test compounds were added 2 to 5 min before addition of agonist (at EC₅₀ value). EC₅₀ is defined as the agonist concentration generating 50% of the peak fluorescence values. IC₅₀ is the concentration required to inhibit 50% of the peak fluorescence.

**Radioligand Binding Studies.** Transiently transfected HEK 293T cells were grown and harvested, and crude membranes were prepared as described (Nuthacker et al., 1999). For competition binding studies, membranes (200 µg of total membrane protein) were incubated with 1.5 nM [³H]LT D₄ (NEN Life Science, Boston, MA) and variable concentrations of competitors in 250 µl of 50 mM Tris-HCl pH 7.4, 20 mM CaCl₂, 25 mM MgCl₂, 10 mM glycine, and 10 mM cysteine for 1 h at 22°C. The membranes were filtered over Whatman GF/C filters using a Brandel cell harvester, washed thrice with ice-cold binding buffer, and counted for 60% of total binding. Data were analyzed by nonlinear regression analysis using PRISM software (GraphPad).

**RNA-Array and Northern Blot Analysis.** Human multiple tissue arrays (CLONTECH) were analyzed by hybridization to an

<table>
<thead>
<tr>
<th>Agonist</th>
<th>CysLT₁ EC₅₀ (nM)</th>
<th>HPN321 EC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTC₄</td>
<td>43 ± 4</td>
<td>8.9 ± 1.4</td>
</tr>
<tr>
<td>LTD₄</td>
<td>0.9 ± 0.1</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>LTE₄</td>
<td>104 ± 6</td>
<td>293 ± 37</td>
</tr>
<tr>
<td>BAY u9773</td>
<td>&gt;10,000</td>
<td>92 ± 15</td>
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</tbody>
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**Antagonist**

<table>
<thead>
<tr>
<th>IC₅₀ (nM)</th>
<th>CysLT₁ IC₅₀ (nM)</th>
<th>HPN321 IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAY u9773</td>
<td>440 ± 182</td>
<td>300 ± 92</td>
</tr>
<tr>
<td>Zafirlukast</td>
<td>0.26*</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Montelukast</td>
<td>2.3*</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>MK-571</td>
<td>10.4*</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>LY-17883</td>
<td>ND</td>
<td>&gt;5000</td>
</tr>
</tbody>
</table>

*IC₅₀ values were determined relative to a 10 nM LTD₄ stimulation.

**Sarau et al., 1999**

**Lynch et al., 1999**

**ND, not determined.**
Identification and Molecular Characterization of the CysLT₁-Like Receptor, HPN321. Computational screenings of public expressed sequence tag and genomic databases were carried out to identify novel sequences displaying structural characteristics common to GPCRs. One genomic clone (GenBank accession no. AL137118) contained an intronless open reading frame of 347 amino acids (Fig. 1) exhibiting seven putative membrane-spanning helices characteristic of GPCR sequences and sharing 36% amino acid identity with the recently cloned CysLT₁ receptor (Lynch et al., 1999). This sequence, named HPN321, was amplified by PCR from human genomic DNA, inserted into a mammalian expression vector under the control of a CMV promotor, and transiently expressed in HEK 293T cells. The transfected cells were challenged with either LTC₄, LTD₄, and LTE₄ in a dose-dependent manner, with respective EC₅₀ values of 8.9, 4.4, and 293 nM (n = 3, Fig. 2). No activation could be observed by addition of the non-peptido-leukotriene LTB₄, even at concentrations up to 5 μM. The highest concentrations of LTC₄ and LTD₄ produced similar maximal responses, whereas LTE₄ behaved as a partial agonist, attaining only 60% of the maximal response of that elicited by LTC₄ and LTD₄. Consequently, the rank order of potency was LTC₄ = LTD₄ > LTE₄, distinct from that of the CysLT₁ receptor (Table 1). We also tested several structurally different and selective CysLT₁ antagonists (LY-17883, MK-571, montelukast and zafirlukast) to assess their ability to block HPN321 activation. All of the CysLT₁ antagonists tested were practically inactive, showing only weak inhibition at concentrations of >1 μM (Table 1).

Pharmacological Properties of BAY u9773 at HPN321 and CysLT₁. To further characterize this novel receptor, we tested BAY u9773, a compound reported to behave as a dual CysLT1/CysLT2 antagonist. When applied simultaneously with the agonist LTD₄ (10 nM, in presence of 5 μM BAY u9773) BAY u9773 exhibited no inhibition. We then administered BAY u9773 alone and found that BAY u9773 was able to activate HPN321 with an EC₅₀ of 100 nM, thus more potently than LTE₄ but less potently than LTC₄ or LTD₄. The response was concentration dependent and reached 67% of the maximal LTC₄ and LTD₄ responses (Fig. 3A), indicating that BAY u9773 acts as a partial agonist. We next tested whether BAY u9773 would also antagonize the effects of full agonists such as LTC₄/LTD₄. We therefore
monitored LTD₄ responses obtained 2 min after the addition of BAY u9773 at variable concentrations. BAY u9773 exhibited its intrinsic agonistic effect but in addition inhibited that elicited by LTD₄ in a dose-dependent manner (Fig. 3, B and C). When BAY u9773 was tested on CysLT₁ transfected cells, it had no intrinsic agonistic effect but elicited an antagonistic effect on a subsequent LTD₄ challenge (Fig. 3D). The IC₅₀ values of BAY u9773 on LTD₄ stimulation were very similar for both receptors (Fig. 3B; Table 1) albeit the antagonistic mechanism at both receptors seemed to be different. The most probable explanation for the antagonistic properties of BAY u9773 at HPN321 is that the drug desensitizes the system in a manner similar to that seen by repeated challenge with LTC₄. To provide additional data demonstrating that BAY u9773 is a partial agonist at HPN321 receptor and that all of its effects in HEK 293T cells can be explained by desensitization of [Ca²⁺]i mobilization responses, we carried out a series of mutual desensitization experiments (Fig. 4). Sequential addition of 100 nM BAY u9773 leads to a desensitization of the HPN321 specific response, leaving other calcium mobilization agonists unaltered as shown by the normal response to UTP (Fig. 4B). LTC₄ and LTD₄ applied at an equipotent concentration desensitized a secondary BAY u9773 challenge (Fig. 4, C and D). The observed homologous desensitization events strongly suggest a similar mode of action for the natural agonists as well for BAY u9773.

**Displacement of [³H]LTD₄.** Competition binding experiments carried out on membranes of transiently transfected HEK 293T cells (Fig. 5) revealed the same rank order of potency for the competition of [³H]LTD₄ binding sites as obtained in the functional assays. LTC₄ and LTD₄ showed high binding affinity, with IC₅₀ values of 4 and 7 nM, respectively. In contrast, LTE₄ competed rather weakly, as reflected by its IC₅₀ value of 0.7 μM. BAY u9773 fully inhibited [³H]LTD₄ binding with an IC₅₀ value of 0.4 μM, very similar to the values obtained for the calcium mobilization assay (Table 1).

**Tissue Distribution of HPN321.** Multiple human tissues were examined by Northern hybridization and dot blot analysis to determine sites of HPN321 expression (Fig. 6A). Using a 600-base pair 5′-probe that did not cross-hybridize with CysLT₁-DNA, we found the strongest expression in the adrenal gland, the heart, and the placenta. Cardiac expression could be detected throughout the entire heart, including ventricles, atrium, septum, and apex. Moderate expression could be detected in the immune system, in particular spleen, lymph nodes, and peripheral blood leukocytes. No signals were found in HL-60, a cell line known to express CysLT₁ receptors. We also investigated U-973 myeloid leukemia cells for HPN321 expression because this cell line has been used to develop CysLT₁ antagonists (Sarau et al., 1999). However, in neither undifferentiated nor differentiated U-973 cells could HPN321 expression be detected, whereas CysLT₁ mRNA was easily detected (Fig. 6B). HPN321-expression was present in lower levels throughout the central nervous system, with...
Fig. 6. Tissue distribution of HPN321 receptor mRNA. A, human normalized multiple tissue array. Top, array after hybridization to a 600-base-pair HPN321 specific probe. The diagram shows the type and position of poly(A) RNAs dotted on the membrane. B, Northern blot of total RNA isolated from undifferentiated (basal) and dimethyl sulfoxide-differentiated (differ.) HL-60 or U-937 cells. The identical membrane was either hybridized to a HPN321 (upper) or a CysLT1 (middle) specific probe. Left and right panels in each column correspond to 5 and 15 µg of total RNA, respectively. HEK cells stably expressing HPN321 were used as a positive hybridization control.
highest levels in pituitary and spinal cord. This expression pattern is distinct from that of the CysLT₁ receptor (Lynch et al., 1999; Sarau et al., 1999) yet overlapping in some tissues.

**Discussion**

While searching in silico for novel GPCRs, we discovered a DNA sequence that could encode a putative GPCR sharing moderate sequence similarity (36%) with the recently described CysLT₁ receptor. When expressed in HEK 293T cells, this new receptor responded to low concentrations of CysLTs by inducing intracellular calcium mobilization. Furthermore, this receptor was not inhibited by antagonists specific to the CysLT₁ receptor, fitting the postulated pharmacological profile of CysLT₂ receptor.

Interestingly, this receptor could be partially activated by the leukotriene analog BAY u9773, originally described as a dual CysLT₁/CysLT₂ antagonist. In this report we demonstrate that BAY u9773 acts as a partial agonist at the new receptor and that this property may have led to its classification as a CysLT₂ antagonist. BAY u9773 has been a useful tool to define CysLT₂ receptors pharmacologically. It has been shown to inhibit physiological responses insensitive to CysLT₁ antagonists in several species including man (Labat et al., 1992; Tudhope et al., 1994; Bäck et al., 1996), thus allowing the detection of the CysLT₂ sites.

A functional model of CysLT₁ and CysLT₂ receptors in human lung has been developed (Gorenne et al., 1996). CysLT₁ receptors, mainly located in bronchial smooth muscle, mediate the contractions evoked by CysLTs. In contrast, CysLT₂ receptors located mainly in the vascular smooth muscles of pulmonary veins, induce contractions that cannot be blocked by CysLT₁ specific antagonists. In addition, the vascular endothelium contains both receptor subtypes, a CysLT₁ type associated with contractions and a CysLT₂ type associated with relaxation. We were able to demonstrate an important pharmacological difference in the action of BAY u9773 at CysLT₁ and CysLT₂ receptors, which enables us now to explain previous findings. Labat et al. had already reported that BAY u9773 elicits small contractile responses in human pulmonary veins and speculated that this effect was caused by a partial agonist activity (Labat et al., 1992). We show that BAY u9773 acts as a partial agonist at HPN321 receptor and thus suggest that it represents the target of BAY u9773 in the human pulmonary venous preparation. In addition, the same authors observed a BAY u9773-induced relaxation in human lung tissues. We can therefore speculate that the same receptor exists in human lung coupled to a relaxation effect, probably mediated through stimulation of endothelial nitric-oxide synthase. BAY u9773 was also reported to contract various tissue preparations from guinea pig (Tudhope et al., 1994; Wikström Jonsson et al., 1998). It must be emphasized, however, that the molecular targets of BAY u9773 in this species might not be identical to the CysLT receptors found in the human lung (Gorenne et al., 1996).

One recent study in guinea pig lung parenchyma found that contractions evoked by BAY u9773 were antagonized by a CysLT₁ specific antagonist (Wikström Jonsson et al., 1998). This result suggests the existence of a CysLT₁-like molecular target for BAY u9773 in this guinea pig tissue. However, in our hands, the human CysLT₁ receptor (Lynch et al., 1999; Sarau et al., 1999) could not be activated by BAY u9773 and was sensitive to CysLT₁ antagonists. Our results thus support the existence of a pharmacological heterogeneity of CysLT receptors in different species.

Our finding that BAY u9773 acts as a subtype selective agonist offers a unique opportunity to study HPN321 receptor selective physiological activities, particularly in tissues in which HPN321 is dominantly expressed over CysLT₁, such as the adrenal gland and the heart. The antagonistic effects seen by pretreatment with BAY u9773 at CysLT₁ sites might be caused by receptor desensitization. Calcium mobilization responses mediated by HPN321 can be desensitized by repeated challenges of LTC₄, LTD₄, LTE₄, and BAYu9773 as well. Because BAY u9773 is structurally related to LTE₄, a partial agonist at both receptors, it probably competes with the full agonists LTC₄ and LTD₄ for the same binding site. We are currently studying the mechanism of BAY u9773 in greater detail, particularly the surprising agonistic selectivity toward HPN321.

The distribution data of HPN321 suggests major role(s) for this receptor in endocrine and cardiovascular systems. CysLTs are well known for their modulatory effects in cardiovascular functions, where they reduce myocardial contractility and coronary blood flow (Letts and Piper, 1982) and have vasoactive effects (Drazen et al., 1980). They are thus considered to be important players in cardiovascular diseases (for review, see Folco et al., 2000). The strong expression of HPN321 in adrenal gland points at a new tissue where to study the influence of CysLTs on endocrine circuits. Finally, leukotrienes have also been found to act on the pituitary to modulate the release of the pituitary hormones (Hulting et al., 1984; Saadi et al., 1990). Our discovery of the existence of the HPN321 message in pituitary adds a molecular credence to this concept. The HPN321 receptor may thus modulate a variety of different physiological functions, which can now be tested using BAY u9773.

During the preparation of this manuscript, two groups also reported the pharmacological characterization of a second human CysLT receptor (Heise et al., 2000; Takasaki et al., 2000). The sequences of the reported receptors (GenBank accession nos. AF254664 and AB038269) are identical with the one described herein. Relative potencies of the CysLTs are very similar, yet none report the selective activity of BAY u9773.

In summary, we present the molecular and pharmacological characterization of a novel human CysLT receptor subtype that we have named the CysLT₂ receptor and report the tissue distribution of its expression. The receptor shows a selective rank order of potency toward CysLTs and is the most preferred target for LTC₄. We also identified BAY u9773 as a subtype selective agonist for the CysLT₂ receptor and suggest the use of BAY u9773 as a selective tool in studies on the physiological roles of the CysLT₂ receptor in cardiac, neuronal, endocrine, and inflammatory circuits.

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**References**


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