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(54) Title: HUMAN CYSTEINYL LEUKOTRIENE RECEPTOR

(57) Abstract: The present invention relates to a purified cysteinyl leukotriene receptor that is responsive to LTC4 and LTD4 but is not blocked by MK-571. This CysLT2 receptor is used to screen for potential human therapeutic agents.
Human Cysteinyl Leukotriene Receptor

US Government Rights

This invention was made with United States Government support under Grant Nos. R01 GM52722 and T32 GM07055, awarded by National Institutes of Health. The United States Government has certain rights in the invention.

Field of the Invention

The present invention is directed to cysteinyl leukotriene receptors. These receptors are G-protein coupled receptors that transmit cell signaling from the cysteinyl leukotrienes LTC4 and LTD4 to induce contractile activity in smooth muscle cells. More particularly, the present invention is directed to the human cysLT type 2 receptor, HG57, nucleic acid sequences encoding that receptor and antibodies against the HG57 receptor.

Background of the Invention

Cysteinyl leukotrienes (also called peptidyl or sulfidopeptidyl leukotrienes) are eicosanoids that are potent contractile agonists for many smooth muscle types. Of particular importance is the role of cysteinyl leukotrienes in the lung as these eicosanoids are heavily implicated in the pathogenesis of bronchial asthma. Indeed, blockers of leukotriene synthesis (e.g. zileuton (Zyflo™)) or cysteinyl leukotriene receptor antagonists (e.g. montelukast (Singulair™), zafirlukast (Accolate™)) are used clinically for asthma prophylaxis.

Cysteinyl leukotrienes are formed from arachidonic acid by the successive actions of 5-lipoxygenase, leukotriene A4 synthase and a microsomal glutathione-S-transferase. The resultant leukotriene C4 (LTC4) is converted further to LTD4 by γ-glutamyl transpeptidase and to LTE4 by a peptidase. LTC4 and LTD4 are the active products of the pathway while LTE4 is a degradation product.

LTC4 and LTD4 signal cells through G protein-coupled receptors. The cysteinyl leukotriene receptor nomenclature designation is ‘cysLTx’, where x refers to discrete receptors that are defined by agonist/antagonist profiles and, ultimately, by molecular cloning. The cloning and characterization of a cysteinyl leukotriene
receptor was first described in Lynch et al. (1999) *Nature* 399:789. This clone was shown to be the cysLT₁ receptor because it (1) has high affinity for LTD₄, (2) has high affinity for the cysLT₁ antagonists zafirlukast, montelukast and MK-571 and (3) is expressed in airway smooth muscle. However, there exist tissue responses (e.g., pulmonary vein) to cysteiny1 leukotrienes that are not blocked by the prototypical antagonist, MK-571. This alternative receptor, which is named cysLT₂, is blocked by the non-selective compound BAY u9773 but has not been described by molecular cloning. There might exist one or more additional cysteiny1 leukotriene receptors but these, if they exist, have not been well defined by existing pharmacologic tools nor have they been identified by molecular cloning.

**Summary of the Invention**

The present invention is directed to the cysLT₂ cysteiny1 leukotriene receptor (HG57) and nucleic acid sequences encoding for that protein. The receptor can be used to identify ligands that bind to HG57 and function as agonists or antagonists of receptor activity. In particular, antagonists of HG57 cysteiny1 leukotriene receptor function can be used to treat bronchial asthma. In addition, the gene sequences encoding the HG57 cysteiny1 leukotriene receptor and the receptor itself can be used to treat diseases relating to defective HG57 cysteiny1 leukotriene receptor function. Finally antibodies raised against the HG57 cysteiny1 leukotriene receptor can be used as diagnostic tools.

**Brief Description of the Drawings**

Fig. 1: Alignment of cysLT-like receptor (HG57, RSPBT32 and HG55) amino acid sequences. Amino acid residues conserved in all three proteins are indicated by the symbol *.  

Figs. 2A-D: Responses of HG57, HG58 and HG57 + HG57 mRNA injected oocytes to cysteiny1 leukotrienes; Fig. 2A represents calcium-dependent chloride conductance from HG57 (CysLT₁) receptor cRNA-injected *Xenopus laevis* oocytes challenge by LTD₄; Fig. 2B represents calcium-dependent chloride conductance from control oocytes injected with saline and challenged with LTD₄; Fig 2C represents calcium-dependent chloride conductance from HG57 (CysLT₂) receptor
cRNA-injected *Xenopus laevis* oocytes challenge with LTD₄ and LTC₄, and HG57 (CysLT₂) receptor cRNA-injected *Xenopus* oocytes preincubation with pertussis toxin (PTX); Fig. 2D represents calcium-dependent chloride conductance from HG57 (CysLT₂) receptor cRNA-injected *Xenopus laevis* oocytes pretreated with MK-571 or BAY u9773.

**Detailed Description of the Invention**

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, "nucleic acid," "DNA," and similar terms also include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

The term "peptide" encompasses a sequence of 3 or more amino acids wherein the amino acids are naturally occurring or synthetic (non-naturally occurring) amino acids. Peptide mimetics include peptides having one or more of the following modifications:

1. peptides wherein one or more of the peptidyl --C(O)NR-- linkages (bonds) have been replaced by a non-peptidyl linkage such as a --CH₂-carbamate linkage (--CH₂OC(O)NR--), a phosphonate linkage, a -CH₂-sulfonamide (-CH₂-S(O)₂NR--) linkage, a urea (--NHC(O)NH--) linkage, a --CH₂-secondary amine linkage, or with an alkylated peptidyl linkage (--C(O)NR--) wherein R is C₁-C₄ alkyl;

2. peptides wherein the N-terminus is derivatized to a --NRR₁ group, to a --NRC(O)R group, to a --NRC(O)OR group, to a --NRS(O)₂R group, to a --NHC(O)NHR group where R and R₁ are hydrogen or C₁-C₄ alkyl with the proviso that R and R₁ are not both hydrogen;

3. peptides wherein the C-terminus is derivatized to --C(O)R₂ where R₂ is selected from the group consisting of C₁-C₄ alkoxy, and --NR₃R₄ where R₃ and R₄ are independently selected from the group consisting of hydrogen and C₁-C₄ alkyl.

Naturally occurring amino acid residues in peptides are abbreviated as recommended by the IUPAC-IUB Biochemical Nomenclature Commission as follows:
Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Norleucine is Nle; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; Glycine is Gly or G, and X is any amino acid. Other naturally occurring amino acids include, by way of example, 4-hydroxyproline, 5-hydroxylysine, and the like.

Synthetic or non-naturally occurring amino acids refer to amino acids which do not naturally occur in vivo but which, nevertheless, can be incorporated into the peptide structures described herein. The resulting "synthetic peptide" contain amino acids other than the 20 naturally occurring, genetically encoded amino acids at one, two, or more positions of the peptides. For instance, naphthylalanine can be substituted for tryptophan to facilitate synthesis. Other synthetic amino acids that can be substituted into peptides include L-hydroxypropyl, L-3,4-dihydroxyphenylalanyl, alpha-amino acids such as L-alpha-hydroxysyl and D-alpha-methylalanyl, L-alpha.-methylalanyl, beta.-amino acids, and isoquinolyl. D amino acids and non-naturally occurring synthetic amino acids can also be incorporated into the peptides. Other derivatives include replacement of the naturally occurring side chains of the 20 genetically encoded amino acids (or any L or D amino acid) with other side chains.

As used herein, the term "conservative amino acid substitution" are defined herein as exchanges within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues:
   Ala, Ser, Thr, Pro, Gly;

II. Polar, negatively charged residues and their amides:
   Asp, Asn, Glu, Gln;

III. Polar, positively charged residues:
   His, Arg, Lys;

IV. Large, aliphatic, nonpolar residues:
   Met, Leu, Ile, Val, Cys

V. Large, aromatic residues:
   Phe, Tyr, Trp
As used herein, the term "purified" and like terms relate to the isolation of a molecule or compound in a form that is substantially free of contaminants normally associated with the molecule or compound in a native or natural environment.

As used herein, the term "HG57 polypeptide" or "HG57 receptor" and like terms refers to polypeptides comprising SEQ ID NO: 2, biologically active fragments thereof and polypeptides substantially similar to SEQ ID NO: 2.

As used herein, the term "biologically active fragments" or "bioactive fragment" of an HG57 polypeptide encompasses natural or synthetic portions of the full-length receptor that are capable of specific binding to the natural ligand of the native HG57 polypeptide.

As used herein, the term "treating" includes alleviating the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms.

As used herein, an "effective amount" means an amount sufficient to produce a selected effect. For example, an effective amount of a cysteiny1 leukotriene receptor antagonist is an amount that decreases the cell signaling activity of the cysteiny1 leukotriene receptor.

As used herein the term "solid support" relates to a solvent insoluble substrate that is capable of forming linkages (preferably covalent bonds) with other compounds. The support can be either biological in nature, such as, without limitation, a cell or bacteriophage particle, or synthetic, such as, without limitation, an acrylamide derivative, agarose, cellulose, nylon, silica, or magnetized particles.

The present invention is directed to cysteiny1 leukotriene receptors that have high affinity for LTC4 and LTD4, but are not blocked by incubation with the prototypical cysLT1 drug MK-571. In accordance with one embodiment, the present invention is directed to the HG57 receptor (SEQ ID NO: 2) and the nucleic acid sequence encoding that receptor (SEQ ID NO: 1).

The HG57 receptor was isolated, based in part, on applicants' recognition of the relevance of an uncharacterized rat nucleotide sequence contained in recently released material in the Genbank database. The identified rat DNA
sequence encoded a partial protein that was about 40% identical to the human cysLT₁ receptor amino acid sequence. This rat DNA sequence was used to isolate a full length rat cDNA sequence, that in turn when used to query the Genbank DNA databases further, led to the design of oligonucleotide primers that led ultimately to the isolation of a human genomic DNA fragment encoding a 346 amino acid protein. This protein is most closely related to cysLT₁ (37% identical amino acids), and its corresponding DNA (or its transcribed RNA), when introduced into cultured human embryonic kidney cells or amphibian (Xenopus) oocytes confers responsiveness (calcium mobilization) to LTD₄. These responses are not blocked by incubation with MK-571, indicating that this clone, although a cysteinyi leukotriene receptor, is not a cysLT₁-type receptor. Interestingly, the responses, including radioligand binding, appear more robust when the new cysLT receptor (HG57) and the existing cysLT₁ receptor are expressed together. Accordingly, the data indicates that the presently identified sequence (SEQ ID NO: 2) represents a second cysLT receptor and that this receptor interacts with the cysLT₁ receptor.

In one embodiment, the present invention is directed to a purified polypeptide comprising the amino acid sequence of SEQ ID NO: 2, or an amino acid sequence that differs from SEQ ID NO: 2 by one or more conservative amino acid substitutions. More preferably, the purified polypeptide comprises an amino acid sequence that differs from SEQ ID NO: 2 by 10 or less conservative amino acid substitutions, and more preferably by 5 or less conservative amino acid substitutions. Alternatively, the polypeptide may comprise an amino acid sequence that differs from SEQ ID NO: 2 by a single mutation, wherein the single mutation represents a single amino acid deletion, insertion or substitution.

Another embodiment of the present invention encompasses truncated versions of the polypeptide of SEQ ID NO: 2, wherein the polypeptide is translated from an alternative start codon located downstream from the first start codon. For example, the polypeptide may comprise the sequence of SEQ ID NO: 5, or an amino acid sequence that differs from SEQ ID NO: 5 by one or more conservative amino acid substitutions, more preferably, by 10 or less conservative amino acid substitutions. The truncated polypeptide of SEQ ID NO: 5 has been expressed in cells and has been found to be functional.
In one embodiment of the present invention the HG57 polypeptide is used to isolate ligands that bind to the HG57 polypeptide under physiological conditions. The method comprises the steps of contacting the HG57 polypeptide with a mixture of compounds under physiological conditions, removing unbound and non-specifically bound material, and isolating the compounds that remain bound to the HG57 polypeptides. Typically, the HG57 polypeptide will be bound to a solid support using standard techniques to allow rapid screening compounds. The solid support can be selected from any surface that has been used to immobilize biological compounds and includes but is not limited to polystyrene, agarose, silica or nitrocellulose. In one embodiment the solid surface comprises functionalized silica or agarose beads. Screening for such compounds can be accomplished using libraries of pharmaceutical agents and standard techniques known to the skilled practitioner.

Ligands that bind to the HG57 polypeptide can then be further analyzed for agonist and antagonist HG57 receptor activity as described in the Examples. Thus in accordance with one embodiment, the HG57 receptor is used to screen chemical libraries for potential LTD4 mimetics and receptor blockers, to allow the development of new chemical entities with therapeutic value. Such compounds can then be formulated as pharmaceutical compositions and administered to a subject to treat bronchial asthma and other disorders associated with aberrant cysteinyll leukotriene receptor signaling.

In accordance with one embodiment of the present invention a method for screening for potential human therapeutic agents is described. The method comprises contacting the HG57 receptor of SEQ ID NO: 2 with a candidate compound, and determining if the candidate compound binds to said receptor. For example, the receptor can be linked to a solid surface using standard techniques and the bound receptors contacted with one or more compounds to determine which compounds are capable of binding. Alternatively, the genes encoding the cysteinyll leukotriene receptor can be expressed in various cell lines. The cellular effects of a variety of compounds that bind to the expressed cysteinyll leukotriene receptor on the surface of the cells can be investigated to provide greater insight on potential in vivo therapeutic effects. The HG57 polypeptide and the gene encoding that protein can also be used as a diagnostic agent for identifying human bronchial asthma cases and
characterizing the severity of each case to allow more effective therapeutic regimens.

The present invention also encompasses nucleic acid sequences that encode the HG57 polypeptide, and bioactive fragments and derivatives thereof. In particular the present invention is directed to nucleic acid sequences comprising the sequence of SEQ ID NO: 1 or fragments thereof. In one embodiment, purified nucleic acids comprising at least 8 contiguous nucleotides (i.e., a hybridizable portion) that are identical to any 8 contiguous nucleotides of SEQ ID NO: 1 are provided. In other embodiments, the nucleic acids comprises at least 25 (contiguous) nucleotides, 50 nucleotides, 100 nucleotides, 200 nucleotides, or 500 nucleotides of SEQ ID NO: 1.

In one embodiment the nucleic acid sequence comprises a 350 bp nucleic acid sequence that is identical to a contiguous 350 bp sequence of SEQ ID NO: 1.

The present invention also includes nucleic acids that hybridize (under conditions defined herein) to all or a portion of the nucleotide sequence represented by SEQ ID NO:1 or its complement. The hybridizing portion of the hybridizing nucleic acids is typically at least 15 (e.g., 20, 25, 30, or 50) nucleotides in length. The hybridizing portion of the hybridizing nucleic acid is at least 80%, and more typically at least 95%, or 98%, identical to the sequence of a portion or all of the nucleic acid of SEQ ID NO:1. Hybridizing nucleic acids of the type described herein can be used, for example, as a cloning probe, a primer (e.g., a PCR primer), or a diagnostic probe. It is anticipated that the DNA sequence of SEQ ID NO: 1, or fragments thereof can be used as probes to detect additional cysteine leukotriene receptor genes, including additional members of the cysteine leukotriene receptor families.

Nucleic acid duplex or hybrid stability is expressed as the melting temperature or Tm, which is the temperature at which a nucleic acid duplex dissociates into its component single stranded DNAs. This melting temperature is used to define the required stringency conditions. Typically a 1% mismatch results in a 1°C decrease in the Tm, and the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if two sequences having > 95% identity, the final wash temperature is decreased from the Tm by 5°C). In practice, the change in Tm can be between 0.5°C and 1.5°C per 1% mismatch.

The present invention is directed to the nucleic acid sequence of SEQ ID NO: 1 and nucleic acid sequences that hybridize to that sequence under stringent or
highly stringent conditions. In accordance with the present invention highly stringent conditions are defined as conducting the hybridization and wash conditions at no lower than -5°C Tm. Stringent conditions are defined as involve hybridizing at 68°C in 5x SSC/5x Denhardt's solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at room temperature. Moderately stringent conditions include washing in 3x SSC at 42°C. Additional guidance regarding such conditions is readily available in the art, for example, by Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.) at Unit 2.10.

In another embodiment of the present invention, nucleic acid sequences encoding the HG57 receptor can be inserted into expression vectors and used to transfect cells to enhance the expression of those receptors on the target cells. In accordance with one embodiment, nucleic acid sequences encoding HG57, or a fragment or derivative thereof, are inserted into a eukaryotic expression vector in a manner that operably links the gene sequences to the appropriate regulatory sequences, and the HG57 receptor is expressed in a eukaryotic host cell. Suitable eukaryotic host cells and vectors are known to those skilled in the art. In particular, nucleic acid sequences encoding HG57 may be added to a cell or cells in vitro or in vivo using delivery mechanisms such as liposomes, viral based vectors, or microinjection.

Accordingly, one aspect of the present invention is directed to transgenic cell lines that contain recombinant genes that express the HG57 receptor. As used herein a transgenic cell is any cell that contains a nucleic acid sequence that has been introduced into the cell and is sufficiently stable to be passed on to progeny cells. In one embodiment the transgenic cell comprises the nucleic acid sequence of SEQ ID NO: 1, or a fragment thereof, wherein the fragment encodes a bioactive fragment of the receptor. In another embodiment the transgenic cell comprises a nucleic acid sequence that hybridizes to at least a portion of SEQ ID NO: 1 under stringent or highly stringent conditions.

In one embodiment the transgenic cell is a eukaryotic cell and the nucleotide sequences encoding the HG57 receptor are operably linked to regulatory sequences that allow expression of the gene in a eukaryotic cell. Such regulatory
sequences are well known to those skilled in the art. In one preferred embodiment, the transgenic cell is a RH7777 or a HEK293T cell. The present invention also encompasses transgenic cell lines that comprise the gene sequences of both the cysLT₁, and the cysLT₂ (HG57) genes. Cells expressing one or both of the cysLT₁, and HG57 genes will allow for the study of the interactions of these two receptors. The use of these transgenic cell lines will allow the identification new pharmaceuticals whose therapeutic effect derives from the synergistic effects of the two receptors.

Transgenic cells comprising the HG57 receptor of the present invention are used in accordance with one embodiment of the invention in assays to detect antagonists and agonists of HG57 receptor activity. Furthermore, as an alternative to working with live cells, extracts of these cells can be prepared and the isolated membranes can be used in assays to detect agonist and antagonists of HG57 receptor activity using the techniques described in the Examples. Thus in accordance with one embodiment, the present invention is directed to membranes isolated from extracts of the transgenic cells, wherein the isolated membranes comprise recombinant HG57 receptors. In one preferred embodiment, the recombinant HG57 receptor is a human HG57 receptor, embedded in the isolated membranes, and comprises the sequence of SEQ ID NO: 2 or an amino acid sequence that differs from SEQ ID NO: 2 by one or more conservative amino acid substitutions.

Another embodiment of the present invention comprises antibodies that are generated against the HG57 receptor. These antibodies can be formulated with standard carriers and optionally labeled to prepare therapeutic or diagnostic compositions. Antibodies to the HG57 receptor may be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and fragments produced by a Fab expression library. These antibodies can be used as diagnostic agents for the diagnosis of conditions or diseases characterized by expression or overexpression of HG57, or in assays to monitor patients being treated with HG57 receptor agonists, antagonists or inhibitors. The antibodies useful for diagnostic purposes may be prepared in the same manner as those described above for therapeutics. The antibodies may be used with or without modification, and may be
labeled by joining them, either covalently or non-covalently, with a reporter molecule.

Example 1

Identification and Characterization of the HG57 receptor

Materials. LTD₄ and LTC₄ were from Cayman (Ann Arbor, MI); LTB₄, LTE₄, and BAY u9773 were from BIOMOL (Plymouth Meeting, PA); 1-oleoyl lysophosphatidic acid was from Avanti Polar Lipids (Alabaster, AL). MK-571, montelukast, pranlukast and zafirlukast were synthesized by the Department of Medicinal Chemistry at Merck Frosst, and [³H]LTD₄ (146 Ci/mmol) was from NENÔ Life Science Products (Boston, MA).

Cloning of HG57, the CysLT₁ receptor. Using the FAST_PAN program (Retief J, Lynch KR and Pearson WR (1999) Genome Res. 9, 373-382), we identified in the EST division of GenBank a rat DNA sequence (accession number ai178926) encoding a partial protein that shared about 40% identical amino acids with the human cysLT1 receptor. The underlying cDNA (clone_ID_RSPBT32) was obtained from the American Type Culture Collection and, after verifying its sequence, it was used to isolate a full length cDNA from a rat brain cDNA library. The full length rat cDNA was subcloned and tested repeatedly for responses to leukotrienes and other lipid mediators (e.g. 5-oxo-ETE). The rat RSPBT32 clone failed uniformly in the assay systems, and thus it remains an 'orphan' G protein-coupled receptor.

Nevertheless, the RSPBT32 amino acid sequence was incorporated into the FAST_PAN query set and subsequently identified a 319 bp human DNA sequence in the human genome survey sequence (GSS) division of GenBank (accession number aq001459) encoding a partial protein that is about 75% identical to RSPBT32. This human sequence information was used to design oligonucleotide primers for the PCR reaction that was used to identify and clone a fragment of human genomic DNA. This human genomic DNA fragment was used to screen a human genomic library, from which a 3.3 kbp SacI fragment was isolated. Sequence analysis of the cloned DNA showed it contains a translational open reading frame (ORF) of 1044 nucleotides (347 amino acids) (SEQ ID NO:2). This translational open reading frame was amplified using PCR and subcloned into the TA cloning site of the mammalian expression vector, pCR3.1 (Invitrogen, Carlsbad, CA). Its encoded
protein, which was named HG57, is 73% identical to rat RSPBT32 and 37% identical to human cysLT1 (originally known as HG55). The next most similar protein (as assessed by FASTA) is the orphan GPCR, humGPR17, which is 32% identical to HG57.

Since a genomic DNA clone was isolated rather than cDNA, it remains uncertain whether an intron interrupts the 5′ region of the ORF, and thus an absolute determination of the codon that is the initiation methionine cannot be made. As can be seen from the alignment of the HG57, RSPBT32 and human cysLT1 sequences (Fig. 1), it is the third methionine in the HG57 open reading frame that aligns with the initial methionine of both RSPBT32 and human cysLT1.

_**X. laevis oocyte expression.**_ HG57 (CysLT2) cRNA was produced by _in vitro_ transcription (T7 mMessage mMMachine, Ambion) using T7 RNA polymerase in the presence of a capping analogue. Twenty to forty ng of the capped cRNA was injected into stage V-VI _X. laevis_ oocytes as described previously (Am. J. Physiol. (1993), 264, C1360-C1364). Compounds were delivered as a 30 μl aliquot over a period of 1-2 sec and the recording chamber washed with 30% methanol between oocyte assays to remove residual ligand. _X. laevis_ were purchased from Xenopus I (Ann Arbor, MI).

**Aequorin Luminescence Functional Assay.** Human embryonic kidney (HEK) 293 cells stably expressing the SV40 large T antigen, designated HEK 293T cells, were transfected with HG57 (CysLT2)-pCR3.1 or pCR3.1 and AEQ-pCDM plasmids (5 μg of each DNA per 75 cm² culture flask), using the LipofectAMINE™ PLUS reagent ( Gibco-BRL) following the manufacturer’s instructions. Cells were prepared subsequently for use in the aequorin luminescence functional assay as described previously (Anal. Biochem. (1999) 272, 34-42). Briefly, agonists in dimethylsulfoxide (DMSO) or ethanol were serially-diluted, in duplicate, in a white 96-well cliniplate FB (Labsystems) using a Biomek in a final volume of 100 μl in PBS (with 70 mg CaCl₂) so that the final solvent concentration was ≤ 1%. The plate was then loaded into the Luminoskan RS plate reading luminometer (Labsystems, Needham Heights, MA), and wells were tested sequentially. Cells (~2-5 x 10⁴ in 100 μl Ham’s F12 medium, were injected into the well and light emission was recorded over 30 s (Peak 1). The cells were then lysed by injection of 25 μl of 0.9% Triton-X 100 solution in H₂O, and light emission measured for an additional 10 s (Peak 2). For
the antagonist, the plate was divided into four series. Series 1 contained a leukotriene C₄ or leukotriene D₄ control curve into which transfected cells were injected in the absence of the antagonist, while series 2 through 4 contained leukotriene C₄ or leukotriene D₄ curves, into which cells, that had been preincubated for 15 min in the presence of a given concentration of antagonist, were injected.

Fractional luminescence for each well was determined by dividing the area under peak 1 by the total area under peaks 1 and 2 (P1/(P1+P2)). These calculations were performed using the Lskan Controller program, and data files were analyzed using the LDAM software employing a modified version of the Levenberg-Marquardt four-parameter curve fitting algorithm to calculate EC₅₀ values (14). For the antagonist tests, the EC₅₀ values were then used to generate a Kᵦ value using Schild plot analysis.

**Radioligand Binding Assays.** Cos-7 cell transfection, harvesting and membrane preparation were done as previously reported (Eur. J. Pharmacol. (1993) 244, 239-250). Tritiated LTD₄ binding assays were performed as described (Eur. J. Pharmacol. (1993) 244, 239-250) with the exception that the reaction was initiated by addition of 250 μg membrane protein and that 4 mM acivicin replaced serine-borate in equilibrium competition assays with LTC₄. LTD₄ specific binding was calculated by subtracting non-specific binding, determined in the presence of 1 μM LTD₄ from total binding. Specific binding accounted for 50-60% of the total binding and was linear with respect to the concentrations of radioligand and protein present in the incubation. Total binding represented less than 10% of the radioligand added to the incubation. In these conditions, average total and non-specific binding of [³H]LTD₄ for 250 μg of HG57 (CysLT₂) receptor membrane protein was 2,600 and 1,200 dpm, respectively.

**Northern Blot Analysis.** RNAs from several human tissues were extracted. Total RNA was extracted by the method of Chomczynski and Sacci and poly (A)+ RNA isolated using oligo(dT) cellulose spin columns (Pharmacia, Uppsala, Sweden). RNA was denatured and size fractionated on a 1% formaldehyde agarose gel, transferred onto nylon membrane and immobilized by UV irradiation. The blots were hybridized with a [³²P]-labeled DNA fragment encoding the CysLT₁ receptor, washed with 2X SSPE and 0.1% SDS at room temperature for 15 min and again with 0.5X SSPE and 0.1% SDS at 50°C for 45 min and four final washings with 0.2 X SSPE and
0.1% SDS at 50 °C each for 1 hour and exposed to X-ray film at -70 °C in the presence of an intensifying screen for 3-7 days.

**Results**

5  **Sequence comparison** Phylogenetic analysis showed that the human orphan GPCR HG57 (identified here as the CysLT₁ receptor), was 73% identical to the rat orphan GPCR RSBPT32 sequence, 38% identical to the human CysLT₁ receptor sequence and 33-35% identical to the human orphan GPCRs GPR17 and GPR23. Despite it’s high similarity to HG57, CysLT activation of the rat RSBPT32 receptor has not been demonstrated.

10  **Xenopus oocyte functional activation** The CysLTs LTD₄ and LTC₄ produced calcium-dependent chloride flux in HG57 (CysLT₂) receptor cRNA-injected *Xenopus laevis* oocytes and were desensitized to subsequent challenge by CysLTs (Fig. 2A). Control oocytes injected with saline or other GPCR cRNAs, including those encoding RSPBT32 and GPR17, showed no response to LTD₄ or LTC₄ but were able to respond to lysophosphatidic acid (LPA) challenge through an endogenous LPA receptor (Fig. 2B). LTD₄ and LTC₄ produced dose-dependent activation of calcium-dependent chloride flux in HG57 (CysLT₂) receptor cRNA-injected *Xenopus* oocytes and the maximal response was not significantly reduced by preincubation with pertussis toxin (Fig. 2C) indicative that CysLT2 did not couple to Gₐ₅ in this system. The LTD₄ or LTC₄ activation of calcium-dependent chloride flux in the HG57 (CysLT₂) expressing oocytes was not blocked by the CysLT₁ receptor selective antagonist, MK-571, but was blocked by the dual CysLT₁/CysLT₂ receptor antagonist BAY u9773 (Fig. 2D).

25  **Transient expression and functional activation in mammalian cells** The HG57 (CysLT₂) receptor was expressed in two mammalian cell lines, namely HEK293T and Cos-7, and in both systems CysLT stimulation of the receptor was shown to activate calcium signaling pathways. In aequorin expressing HEK293T cells, LTD₄ and LTC₄ were equipotent agonists, while LTE₄ behaved as a partial agonist and LTB₄ was inactive up to 10 µM (See Table 1). BAY u9773 acted as a non-competitive antagonist of both LTC₄ and LTD₄-challenged CysLT₂ receptor-expressing HEK293T cells. The selective CysLT₁ receptor antagonists, MK-571,
montelukast, zafirlukast and pranlukast showed no significant antagonism of the CysLT<sub>2</sub> receptor up to 1 μM concentration (See Table 1).

**Table 1**: Functional Agonist Activation of the Recombinant Human CysLT<sub>2</sub> receptor, HG57, Expressed in Hek293T Cells Using an Aequorin-based Assay

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; value (nM)</th>
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<tbody>
<tr>
<td>LTC&lt;sub&gt;4&lt;/sub&gt;</td>
<td>67</td>
</tr>
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**Radioligand receptor binding characterization** Saturation analysis of [³H]LTD<sub>4</sub> specific binding to Cos-7 cell membranes expressing HG57 (CysLT<sub>2</sub>) receptor shows that radiolabeled LTD<sub>4</sub> binds with high affinity (K<sub>d</sub> = 4 nM) to a single population of receptor expressed in low abundance (B<sub>max</sub> = 0.6 pmol/mg membrane protein). In equilibrium competition assays, the rank order of potency of leukotriene agonists to compete with [³H]LTD<sub>4</sub> for binding to the CysLT<sub>2</sub> receptor was LTD<sub>4</sub> = LTC<sub>4</sub> >> LTE<sub>4</sub> with no competition up to 10 μM by LTB<sub>4</sub> (See Table 2). The CysLT<sub>1</sub> receptor antagonists montelukast and zafirlukast, up to 5 μM, did not compete for radiolabeled LTD<sub>4</sub> binding to the CysLT<sub>2</sub> receptor concentration, while pranlukast showed weak competition with an IC<sub>50</sub> value of 3.6 μM (Table 2). However, competition was observed with the dual CysLT<sub>1</sub>/CysLT<sub>2</sub> antagonist BAY u9773 with an IC<sub>50</sub> value of 0.6 μM (Table 2).
Table 2: Radioligand ([3H]LTD4) Binding to COS-7 Cell Membranes Expressing the Recombinant Human CysLT2 receptor, HG57

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Example 2

Human Tissue RNA Northern Blot and In Situ Analysis

The human CysLT2 receptor has been shown to be expressed in peripheral blood leukocytes, lymph nodes, spleen, heart and several central nervous system regions (as disclosed in J. Biol. Chem 275(39) pp 30531-30536 (2000), the disclosure of which is incorporated herein). In human RNA dot blot analysis, the receptor was also shown to be expressed in the adrenal gland. In contrast to the CysLT1 receptor, that is most highly expressed in human lung smooth muscle, the strongest lung expression of the cysLT2 receptor is seen in interstitial macrophages, with distinctly weaker expression is smooth muscle cells. Particularly elevated expression of the CysLT2 receptor as observed in macrophages when in close proximity to smooth muscle cells. Peripheral blood leukocytes were another abundant source for CysLT2 receptor transcripts. In situ analysis of the CysLT2 receptor in partly purified normal human peripheral blood monocytes demonstrated abundant expression in greater than 20% of these cells. In addition, strong expression of the receptor in purified human eosinophils was also observed, a cell type previously reported as expressing the CysLT1 receptor. Tissues other than myeloid cell containing organs, such as the spleen and lymph nodes, that showed expression of the mRNA for the CysLT2 receptor included regions of the CNS, the adrenal gland and the heart.
Claims:

1. A purified cysteiny1 leukotriene receptor that is responsive to LTC4 and LTD4 but is not blocked by MK-571.

2. The cysteiny1 leukotriene receptor of claim 1 comprising the amino acid sequence of SEQ ID NO: 2.

3. A purified polypeptide encoding a cysteiny1 leukotriene receptor wherein said polypeptide comprises
   the amino acid sequence of SEQ ID NO: 2;
   an amino acid sequence that differs from SEQ ID NO: 2 by one or more conservative amino acid substitutions; or
   an amino acid sequence that differs from SEQ ID NO: 2 by a single mutation, wherein the single mutation represents a single amino acid deletion, insertion or substitution.

4. A nucleic acid sequence comprising the sequence of SEQ ID NO: 1 or a nucleic acid sequence that hybridizes to SEQ ID NO: 1 under stringent conditions.

5. A transgenic host cell comprising the nucleotide sequence of claim 4.

6. The transgenic host cell of claim 5 further comprising a nucleic acid sequence that encodes a CsyLT1 receptor.

7. A human cysteiny1 leukotriene receptor gene comprising a 25 bp nucleic acid sequence that is identical to a contiguous 25 bp sequence of SEQ ID NO: 1.

8. A method of screening for potential human therapeutic agents, said method comprising contacting a CsyLT2 receptor with a candidate compound; and determining if the candidate compound selectively binds to said receptor.

9. The method of claim 8 wherein the CysLT2 receptor is expressed on the surface of a cell.

10. The method of claim 9 wherein the CysLT2 receptor comprises the polypeptide of SEQ ID NO: 2.

11. The method of claim 10 wherein radioligand ([^3]H)LTD4 binding to cell membranes is monitored in the presence and absence of the potential human therapeutic agent.
12. The method of claim 10 wherein the calcium mobilization in response to LTC4 and LTD4 in whole cells is measured in the presence and absence of the potential human therapeutic agent.

13. The method of claim 8 wherein the cell also expresses a cysLT1 receptor.
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FIG. 1
**FIG. 2C**

- Log LTs vs. Cl current (-μA)

**FIG. 2D**

- Log [LTD4]10^{-6}M vs. Cl current (-μA)

- Log [drug]
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Leu Tyr Lys Ile Ala Lys Leu Gln Thr Met Asn Tyr Ile Ala Leu Val  
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Val Ser His Arg Lys Ala Leu Thr Thr Ile Ile Ile Thr Leu Ile Ile  
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Thr Thr Trp Lys Val Gly Leu Cys Lys Asp Arg Leu His Lys Ala Leu  
45 260 265 270
Val Ile Thr Leu Ala Leu Ala Ala Ala Asn Ala Cys Phe Asn Pro Leu
275 280 285

Leu Tyr Tyr Phe Ala Gly Glu Asn Phe Lys Asp Arg Leu Lys Ser Ala
290 295 300

Leu Arg Lys Gly His Pro Gln Lys Ala Lys Thr Lys Cys Val Phe Pro
305 310 315 320

Val Ser Val Trp Leu Arg Lys Glu Thr Arg Val
325 330
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C12P 21/06; C12N 15/63, 15/85, 15/86; G01N 33/53; C12Q 1/04
US CL : 530/350; 536/23.5; 435/69.1; 435/320.1; 435/325; 435/7.1; 435/35

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim</th>
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* Further documents are listed in the continuation of Box C.

See patent family annex.

Date of the actual completion of the international search

12 February 2001 (12.02.2001)

Authorized officer

Sandra Wax

Date of mailing of the international search report

30 MAR 2001

Name and mailing address of the ISA/US

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Telephone No. 703.308-0196

Form PCT/ISA/210 (second sheet) (July 1998)