Pharmacologic Actions of the Second-Generation Leukotriene B₄ Receptor Antagonist LY293111: In Vitro Studies

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ABSTRACT

The in vitro actions were investigated of LY293111, a potent and selective leukotriene B₄ (LTB₄) receptor antagonist, on human neutrophils, human blood fractions, guinea pig lung membranes, and guinea pig parenchymal and tracheal strips. The IC₅₀ for inhibiting [³H]LTB₄ binding to human neutrophils was 17.6 ± 4.8 nM. LY293111 inhibited LTB₄-induced human neutrophil aggregation (IC₅₀ = 32 ± 5 nM), luminol-dependent chemiluminescence (IC₅₀ = 20 ± 2 nM), chemotaxis (IC₅₀ = 6.3 ± 1.7 nM), and superoxide production by adherent cells (IC₅₀ = 0.5 nM). Corresponding responses induced by N-formyl-L-methionyl-L-leucyl-L-phenylalanine were inhibited by 100-fold higher concentrations of LY293111. LTB₄ binding to guinea pig tissues and subsequent activation were also inhibited. The Kᵢ for inhibition of [³H]LTB₄ binding to lung membranes was 7.1 ± 0.8 nM; IC₅₀ for preventing binding of [³H]LTB₄ to spleen membranes was 65 nM. The compound inhibited LTB₄-induced contraction of guinea pig lung parenchyma. At 10 nM, LY293111 caused a parallel rightward shift of the LTB₄ concentration-response curve. At higher concentrations, plots were shifted in a nonparallel manner, and maximum responses were depressed. LY293111 did not prevent antigen-stimulated contraction of sensitized trachea strips. At micromolar concentrations, LY293111 inhibited production of LTB₄ and thromboxane B₂ by plasma-depleted human blood stimulated with N-formyl-L-methionyl-L-leucyl-L-phenylalanine and thrombin. In addition, at these higher concentrations, formation of LTB₄ by A23187-activated whole blood and conversion of arachidonic acid to LTB₄ by a human neutrophil cytosolic fraction were inhibited. In summary, LY293111 is a second-generation LTB₄ receptor antagonist with much improved potency in a variety of functional assay systems.

Leukotriene B₄ (LTB₄) is a lipid mediator initially described by Borgeat and Samuelsson (1979). Borgeat and Nacke (1990) reviewed studies on the biosynthesis and biological activity of the molecule. The molecule, a dihydroxy acid derived from arachidonic acid via the 5-lipoxygenase pathway, activates a number of cell functions; most noteworthy is its ability to promote the unidirectional movement of certain cells such as neutrophils and eosinophils. Other activities include oxidant production, up-regulation of the adhensive tissue CD11b/CD18, secretion of hydrolytic enzymes from granules, enhanced production of suppressor cells, and stimulation of the synthesis and secretion of certain cytokines. Because of these properties, it has been suggested that the eicosanoid may have a pathophysiological role in many inflammatory and immunological diseases such as psoriasis, various arthritides, inflammatory bowel disease, asthma, and certain immunological diseases. The evidence for the role of LTB₄ in these diseases has been documented in several reviews (Ford-Hutchinson, 1990; Lewis et al., 1990; Stenson, 1990; Jackson and Fleisch, 1996). The potential therapeutic value of agents that block the actions of LTB₄ has stimulated several pharmaceutical companies to develop appropriate LT4 receptor antagonists (Sawyer, 1996; Brooks and Summers, 1996) and hydroxyacetophenones (Herron et al., 1992). Using inhibition of the

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ABBREVIATIONS: LTB₄, leukotriene B₄; A23187, 6S-[6α(2S*,3S*),8β(R*)]-9β,11αS-(methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1H)-pyrimidin-2-yl]ethyl]1,7-dioxaspiro[5.5]undec-2-yl]oxy]-4-benzoxazolecarboxylic acid; BES, N,N-bis[2-hydroxyethyl]2-aminoethanesulfonic acid; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; fMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; [³H]LTB₄, [5,6,8,9,11,12,14,15α-³H]leukotriene B₄; LTB₄, leukotriene B₄; LY255283, 1-[5-ethyl-2-hydroxy-4-[6-methyl-6-(1H-tetrazol-5-yl)heptyl]oxy]phe-nyl]ethane; LY293111, 2-[2-propyl-3-[3-2-ethyl-4-(4-fluorophenyl)-5-hydroxyphenoxo]propoxy]phenoxy]benzoic acid; PIPES, piperazine-N,N'- bis[2-ethanesulfonic acid]; SC-41930, 7-[3-[4-acetyl-3-methoxy-2-propoxy]phenoxy]propoxy] [3,4-dihydro-8-propyl-2H-1-benzo[1]benzopyran-2-carboxylic acid; TxA₃, thromboxane A₃; TxB₃, thromboxane B₃; U46619, 15(S)-hydroxy-11α,9α-(epoxymethano)prosta-5,13E-dienoic acid.

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specific binding of radiolabeled LTB₄ to isolated intact human neutrophils as a marker of activity, we found LY223982 [(E)-5-(3-carboxybenzoyl-2-[(6-[4-methoxyphenyl]-5-hexenyl)oxy]benzenepropanoic acid] (IC₅₀ = 13.2 nM) and LY255283 (1-[5-ethyl-2-hydroxy-4-[6-methyl-6-(1H-tetrazol-5-ylhexyl)oxy]phenyl]ethanone) (IC₅₀ = 87 nM) to be the most potent compounds in their respective series. These agents also inhibited various cell functions activated by LTB₄, although IC₅₀ values were generally 10- to 100-fold higher. Unfortunately, the compounds were not very effective when administered orally to guinea pigs undergoing airway obstruction induced by an i.v. injection of LTB₄. LY223982 had no effect when administered as a single 30 mg/kg dose 2 h before the LTB₄ challenge. At high doses (ED₅₀ = 11 mg/kg), LY225283 had some effect (Silbaugh et al., 1992). Thus, because of the poor oral bioavailability, the use of these compounds was restricted primarily to topical application or i.v. injection. Neither route is very useful for an anti-inflammatory drug that must be given chronically. Consequently, we embarked on a program to develop compounds that were more orally bioavailable and more potent in vitro inhibitors of LTB₄-induced cell functions. Initially, our efforts were directed toward modifications of LY223982 because of the two antagonists, it was the more potent in vitro. Xanthone dicarboxylic acids that mimicked different conformation states of benzophenone dicarboxylic acids were evaluated, and one of these (LY210073) was found to be a better inhibitor of LTB₄-stimulated events than LY223982 (Chaney et al., 1992; Jackson et al., 1993). It was, however, not active orally.

We then turned our attention to studying derivatives of LY255283. For this approach, the molecule was divided conceptually into three regions, a lipophilic region containing the 2-hydroxyacetophenone region, an acid region encompassing the tetrazole moiety, and a section linking these two groups. The effect of modifications at either end of the molecule was investigated. The structure-activity studies (Sawyer et al., 1995) eventually led to the discovery of LY293111 (Fig. 1), a compound more potent than LY255283 at selectively inhibiting LTB₄-induced cellular reactions and considerably more active when administered orally than the first-generation antagonist. In this report are described comparative in vitro studies of LY293111, LY255283, and another first-generation LTB₄ receptor antagonist, SC-41930 (7-[3-(4-acetyl-3-methoxy-2-propylphenoxy)propoxy]-3,4-dihydro-8-propyl-2H-1-benzopyran-2-carboxylic acid). In vivo studies on LY293111 will be presented in a later communication.

Materials and Methods

Chemicals. [³H]LTB₄ (specific activity, 150–220 Ci/mmol) was purchased from Amersham (Arlington Heights, IL) or New England Nuclear (Boston, MA). Nonradioactive LTB₄ was obtained from BIOMOL (Plymouth Meeting, PA). A23187 (6S-[6α(2S*,3S*)]-8β(R²)-9β,11α-[5-(methylenamino)-2-[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-[1H-pyrrol-2-yl]ethyl]-1,7-dioxaspiro[5.5]undec-2-yl)methyl]-4-benzoxazolcarboxylic acid), ATP, N,N-bis[2-hydroxyethyl]z3-aminoethanesulfonic acid (BES), bovine serum albumin, cytochalasin B, ethylenediaminetetraacetic acid (EDTA), N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP), [3H]propranolol, piperazine-N,N-bis[2-ethanesulfonic acid] (PIPES), Tris-HCl, and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO).

Inhibition of [³H]LTB₄ Binding to Human Neutrophils. The effectiveness of compounds to inhibit binding of LTB₄ to human neutrophils was measured with a radioligand binding assay. Heparinized venous blood was drawn from normal volunteers, and neutrophils were isolated by standard techniques of Ficoll-Hypaque centrifugation, dextran 70 sedimentation, and hypotonic lysis. Cell preparations were ≥90% viable neutrophils. The binding assay was carried out in silanized 12 × 75-mm glass tubes by adding in the following order: 10 μl of DMSO containing different amounts of antagonist, 20 μl of radioligand (2.65 nM [³H]LTB₄), and 500 μl of cells suspended in Hanks’ balanced salt solution containing 0.1% ovalbumin (2 × 10⁷ cells/ml). Tubes were incubated at 4°C for 10 min, and the reaction was terminated by isolating the cells with a Brandel MB-48R harvester (Gaithersburg, MD). Radioactivity bound to the cells was measured by scintillation spectrometry. Nonspecific binding was determined by measuring the amount of label bound when a greater than 2000-fold excess of nonradioactive ligand was added. Appropriate corrections for nonspecific binding were made when analyzing the data. The concentration of DMSO in the incubation mixture (1.9%) had no effect on the binding of radioligands. The amount of [³H]LTB₄ added (0.1 nM) was only 4% of the IC₅₀ for nonlabeled LTB₄ so that IC₅₀ values obtained are nearly identical with Kᵣ values for the high affinity LTB₄ receptor.

Inhibition of LTB₄-Induced Human Neutrophil Chemotaxis. Studies were carried out using chemotaxis chambers with a 200-μl blind-end stimulus compartment and fitted with 3 μM polyvinylpyrrolidone-free polycarbonate membranes (Poretics Corporation, Livermore, CA). Human polymorphonuclear leukocytes were isolated from citrated venous blood drawn from normal volunteers and resuspended at a density of 12 × 10⁶ cells/ml in Dulbecco’s
phosphate-buffered saline, supplemented with 0.6 mM CaCl2, 1.0 mM MgCl2, 2.0 mM glucose, and 0.05% human serum albumin. The same buffer was used to make stock solutions of LTB4 (20 nM) and antagonists. Equal amounts of a particular stock solution of antagonist and chemotactic agent were mixed, and 200 μl was added to the lower compartment. Equal parts of the human neutrophil suspension and the stock antagonist solution were mixed, and 0.8 ml was added to the upper compartment. Thus, the final concentration of LTB4 in the lower compartment was 10 nM, and a total of 4.8 × 10^6 neutrophils were added to the upper compartment. The chemotaxis chambers were then incubated for 90 min at 37°C. The number of cells that migrated completely through the filter and dropped into the lower compartment was determined with the use of a Sequoia-Turner Cell-Dyn 900 counter (Mountain View, CA). Cells that passed through the filter by chemotaxis were calculated by subtracting, from the total migrated cells, the number that moved by random motion.

**Inhibition of Superoxide Production by Adherent Human Neutrophils.** Cell suspensions in phenol-red-free Hank’s balanced salt solution containing bovine serum albumin (1 mg/ml) were added in 80-μl aliquots (2 × 10^5 neutrophils/well) to 96-well flat-bottom tissue culture plates (Costar, Cambridge, MA). Subsequently, 100-μl aliquots of a ferriyochrome c solution (2.97 mg/ml) in Hank’s balanced salt solution were added to each well containing neutrophils. Ten microliters of test compound at various concentrations were added to each well followed by the addition of 10 μl of stimulant (either LTB4, fMLP, orFMLP). The plates were incubated at 37°C for 30 min after addition of neutrophil stimulant. Absorbance at 550 nM was determined on a micro-ELISA reader (model MR600; Dynatech Laboratories, Alexander, VA). The reference wavelength was set at 490 nm to compensate for changes in absorbance due to the presence of cells (Rajkovic and Williams, 1985). Concentrations of the cytochrome c reduction were controlled by the inclusion in some sample wells of superoxide dismutase (300 U/ml, final concentration). Compounds were initially dissolved in dimethylsulfoxide (DMSO) and then diluted to the desired strength. The concentration of DMSO in the wells never exceeded 0.3%. The results were expressed in nanomoles of cytochrome c reduced per 1 × 10^6 neutrophils after subtraction of absorbance readings for wells containing superoxide dismutase.

**Inhibition of LTB4- and fMLP-Induced Chemiluminescence and Aggregation of Human Neutrophils.** A platelet-activated calcium aggreagomer (Chrono-log Corporation, Havertown, PA) was used to measure chemiluminescence and aggregation of peripheral human neutrophils. Cells (1 × 10^7/ml) suspended in Dulbecco’s phosphate-buffered saline without calcium and magnesium, pH 7.4 (450 μl), were placed in a siliconized cuvette. Cytochalasin B (4.5 μl, 200 μg/ml) was added, and the contents were stirred at 900 rpm and 37°C. After 2 min, agonist (4.5 μl) was injected into the cuvette. Calcium and magnesium ions (4.5 μl, 100 mM CaCl2, 50 mM MgCl2) were then added. After another minute, luminol (4.5 μl, 100 μM) and LTB4 or FMLP (4.5 μl, 3 μM) were injected into the cuvette, and the subsequent maximum amount of response occurring was measured with the aid of a Compaq 386/20e computer and software supplied by Chrono-log Corporation. The final concentration of LTB4 and FMLP was 30 nM. Corrections were made for non-specific chemiluminescence and aggregation occurring in the absence of agonist.

**Inhibition of [3H]LTB4 Binding to Guinea Pig Lung Membranes.** Incubations (555 μl) were performed for 45 min at 30°C in polypropylene minitubes, which contained 25 μg of guinea pig lung parenchyma (Saussey et al., 1995), in a buffer consisting of 25 mM 3-[N-morpholino]propanesulfonic acid, 10 mM MgCl2, 10 mM CaCl2, pH 6.5, approximately 140 pM [3H]LTB4, and displacing ligand or vehicle (0.1% DMSO in 1 mM Na2CO3, final concentration) as appropriate. The binding reaction was terminated by the addition of 1 ml of ice-cold wash buffer (25 mM Tris·HCl, pH 7.5), followed immediately by vacuum filtration over Whatman GF/C glass-fiber filters, using a Brandel (Gaithersburg, MD) 48 place harvester. The filters were washed five times with 1 ml of wash buffer. Retained radioactivity was determined by liquid scintillation counting at 50% counting efficiency using Ready Protein Plus cocktail (Beckman, Fullerton, CA). Nondisplaceable binding was determined in the presence of 1 μM nonradioactive LTB4 and was usually less than 10% of total binding. Data were analyzed using linear regression analysis of log-logit plots of the values between 10% and 90% of control binding to calculate IC50 values and slope factors (pseudo-Hill coefficients). IC50 values thus obtained were used to calculate KJ values according to the method of Cheng and Prusoff (1973).

**Inhibition of [3H]LTB4 Binding to Guinea Pig Spleen Membranes.** Guinea pig spleen LTB4 receptor binding kits were obtained from New England Nuclear (Boston, MA). Displacement of [3H]LTB4 was performed as outlined in the instructions using the supplied reagents. Briefly, 25 μl each of [3H]LTB4, and varying concentrations of LY293111 were incubated with 200 μl of guinea pig spleen LTB4 receptor preparation for 2 h. Bound [3H]LTB4 was separated from free by vacuum filtration, and the KJ for LY293111 was calculated. All steps were carried out at 4°C.

**Inhibition of LTB4-Induced Lung Parenchyma Contraction.** Male Hartley guinea pigs were killed by CO2 suffocation and subsequent decapitation. Lungs were excised and perfused through the pulmonary artery with Krebs-bicarbonate solution of 118.2 mM NaCl, 4.6 mM KCl, 2.5 mM CaCl2·2H2O, 1.2 mM MgSO4·7H2O, 24.8 mM NaHCO3, 1.2 mM KH2PO4, and 10.0 mM dextrose. Strips of parenchyma were removed from the outer edge of the lung, and the ends were secured by cotton thread. The tissues were then placed in 10-ml organ baths under a passive force of 0.5g. Temperature was maintained at 37°C, and the Krebs-bicarbonate solution was aerated with 95% O2/5% CO2. Tissues were equilibrated for at least 1 h before the start of the experiment. Contractions were then induced by increasing concentrations of LTB4 with a washout and 30-min rest period interposed between administrations of the agonist. Contractions were measured isometrically with a Grass PTO3C force-displacement transducer and recorded on a Grass polygraph as changes in g of force. The procedure essentially followed that of Drazen and Schneider (1978). In addition, contractile responses to LTD4, histamine, carbachol, and U46619 were elicited using the cumulative concentration technique of van Rossum (1963). After a control concentration-response curve to each agonist was obtained, the parenchyma was then incubated with LY293111 for 45 min followed by a final concentration-response profile to the respective agonist.

**Effect of LY293111 on Antigen-Induced Contractions of Guinea Pig Tracheal Strips.** Male Hartley strain guinea pigs (200–250 g) were actively sensitized by three 10 mg/kg injections of ovalbumin over a 5-day period, and experiments with tracheal tissue from these animals were performed 21 to 26 days later. On the day of the experiment, guinea pigs were killed by cervical dislocation, and tracheae were removed, cleaned of surrounding connective tissue, and cut into spiral strips. Each strip was divided in half for paired experiments. Tissues were placed in 10-ml jacketed tissue baths maintained at 37°C and attached with cotton thread to Grass force-displacement transducers (PTO3C). Changes in isometric tension were displayed on a Grass polygraph (model 7D). Tracheal strips were bathed in modified Krebs-bicarbonate solution of 118.2 mM NaCl, 4.6 mM KCl, 2.5 mM CaCl2·2H2O, 1.2 mM MgSO4·7H2O, 24.8 mM NaHCO3, 1.0 mM KH2PO4, and 10.0 mM dextrose. The buffer also contained indomethacin (5 μM), which potentiated the contraction of the cysteinyl leukotrienes by removing the influence of cyclooxygenase products. The tissue baths were aerated with 95% O2/5% CO2. Strips were placed under a resting tension of 2g, and the tissues were allowed a minimal stabilization period of 60 min before undergoing experimentation. Bath fluid was changed at 15-min intervals during the stabilization period. Initially, tissues were challenged with carbachol (10 μM) after the 60-min stabilization period to ensure tissue viability. After recording the maximum response to the initial carbachol challenge, the tissues were washed and reequilibrated for 60 min before starting the experimental protocol. Cumulative concentration-response curves were obtained from
tracheal strips by increasing the ovalbumin concentration in the organ bath by half-log\(_2\) increments while the previous concentration remained in contact with the tissues (van Rossum, 1963). Agonist concentration was increased after reaching the plateau of the contraction elicited by the preceding concentration. One concentration-response curve was obtained from each tissue. The paired tissue strips received either LY293111 (10 \(\mu M\)) or vehicle (DMSO) 30 min before starting the cumulative concentration-response curves. To minimize variability between tissues, contractile responses were expressed as a percentage of the maximal response obtained with carbachol (10 \(\mu M\)), added to the bath at the end of the concentration-response curve.

### Inhibition of Eicosanoid Production by Plasma-Depleted Human Blood

Ten milliliters of blood were collected in EDTA (final concentration, 1.5 mg/ml). Then, 40 ml of glucose-phosphate buffer was added, and the suspension was centrifuged at 900g for 10 min at room temperature. The supernatant fluid was discarded, and the cells were washed once with 50 ml of glucose-phosphate buffer before resuspending them in 50 ml of Krebs-Ringer-Henseleit buffer containing 0.1% gelatin, 1 mM CaCl\(_2\), and 1.1 mM MgCl\(_2\). Working solutions of LY293111 were made by dissolving the compound in DMSO at 10 mM and diluting it with appropriate amounts of Krebs-Ringer-Henseleit buffer. An agonist solution was made containing 1 \(\mu M\) FMLP and 10 units/ml of thrombin in Krebs-Ringer-Henseleit buffer. The cell suspension was first warmed at 37\(^\circ\)C for 5 min. For each milliliter of suspension, 2 \(\mu l\) of cytochalasin B solution (2 mg/ml in DMSO) was then added. Experiments were carried out using polypropylene 96-well plates (1 ml well volume). In each well, 200 \(\mu l\) of an appropriate LY293111 solution or buffer was incubated with 250 \(\mu l\) of the blood cell/cytochalasin B suspension at 37\(^\circ\)C for 10 min. Stimulation of eicosanoid production was begun by adding 50 \(\mu l\) of the agonist solution. The mixture was then incubated for 2 min before stopping the reaction by adding 50 \(\mu l\) of a reagent containing 25 mM EDTA, 10 \(\mu M\) indomethacin, and 10 \(\mu M\) concentration of the 5-lipoxygenase inhibitor, LY280810 (Cho et al., 1993). Subsequently, the plate was centrifuged, and an aliquot of the supernatant fluid was removed for analysis of the eicosanoid content. On standing for 1 to 3 h after the centrifugation step, there was a slight amount of red cell hemolysis in the cell pellet when the incubation mixture contained the highest concentration of LY293111 (10 \(\mu M\)) tested. However, this did not interfere with the assay for eicosanoid production because the supernatant fluid samples were removed immediately after centrifugation before the hemolysis occurred. The amount of LTB\(_4\) and TxB\(_2\) produced was determined by the use of a competitive enzyme immunoassay. The IC\(_{50}\) for inhibition of each eicosanoid was determined using blood cells from three individuals and averaging the values.

### Inhibition of Eicosanoid Production in Whole Human Blood

Venous blood was collected into heparin, and a 1-ml aliquot was incubated with LY293111 for 10 min at 37\(^\circ\)C. Eicosanoid production was initiated with the addition of either A23187 (20 \(nM\)) to the cell suspension or 30 nM 5-lipoxygenase inhibitor, LY280810 (Cho et al., 1993). Subsequently, platelet aggregation, and an aliquot of the supernatant fluid was removed for analysis of the eicosanoid content. On standing for 1 to 3 h after the centrifugation step, there was a slight amount of red cell hemolysis in the cell pellet when the incubation mixture contained the highest concentration of LY293111 (10 \(\mu M\)) tested. However, this did not interfere with the assay for eicosanoid production because the supernatant fluid samples were removed immediately after centrifugation before the hemolysis occurred. The amount of LTB\(_4\) and TxB\(_2\) produced was determined by the use of a competitive enzyme immunoassay. The IC\(_{50}\) for inhibition of each eicosanoid was determined using blood cells from three individuals and averaging the values.

### Inhibition of LTB\(_4\) Formation by Human Neutrophil Cytosol

Peripheral blood neutrophils were isolated by density centrifugation as previously described (Marder et al., 1992). The cells were then washed twice in buffer (10 mM PIPES, 10 mM BES, and 1 mM EDTA, pH 6.8), diluted to 2.5 \(\times\) 10\(^7\) cells/ml and disrupted by sonication. The resulting material was subsequently centrifuged at 20,000g for 20 min at 4\(^\circ\)C, and the supernatant fluid was stored at −70\(^\circ\)C until assayed. For testing inhibitory activity, different amounts of LY293111 were added to 150 \(\mu l\) of the cytosol diluted 1:3 (v/v) with assay buffer (10 mM PIPES, 10 mM BES, 1 mM EDTA, 100 mM NaCl, 1.56 mM ATP, and 2.5 mM CaCl\(_2\), pH 6.8). LTB\(_4\) synthesis was initiated by adding 50 \(\mu l\) of a solution of arachidonic acid (final concentration, 3 \(\mu M\)). After a 5-min incubation at 37\(^\circ\)C, the reaction was terminated by the addition of 50 \(\mu l\) of 500 mM EDTA. LTB\(_4\) was determined by enzyme-linked immunoassay using reagents and assay procedures obtained from Cayman Chemical Co. (Ann Arbor, MI).

### Results

#### Inhibition of Specific Binding of \([^{3}\text{H}]\text{LTB}_4\) to Human Neutrophils

The effect of LY293111 on binding of radioactive LTB\(_4\) to its receptors on intact human neutrophils was compared with that of LY255283 and another LTB\(_4\) antagonist, SC-41930, as well as nonradioactive LTB\(_4\) (Fig. 2). The respective IC\(_{50}\) values for the three receptor antagonists were 17.6 ± 4.8 nM for LY293111, 41.3 ± 3.1 nM for SC-41930, and 85.1 ± 7.9 nM for LY255283. Thus, LY293111 is 2.3- and 4.8-fold more potent than SC-41930 and LY255283, respectively, and only 3.8-fold less potent than the homologous ligand (IC\(_{50}\) = 4.6 ± 0.6 nM).

#### Inhibition of Chemotaxis of Human Neutrophils

Figure 3 shows the ability of the three antagonists to inhibit the migration of human neutrophils toward 10 nM LTB\(_4\). LY293111 was considerably more effective at preventing cell movement than the other two compounds. The IC\(_{50}\) for LY293111 (6.3 ± 1.7 nM) was 600 times lower than the corresponding value for SC-41930 (3.8 ± 0.8 nM). LY255283 was the poorest inhibitor of the three (IC\(_{50}\) = 7.2 \(\mu M\)). At 10 \(\mu M\) or less in concentration, LY293111 had no chemotactic activity.

#### Prevention of Oxidant Production by Human Neutrophils

The activity of the antagonists on secretion of reactive oxygen species by stimulated cells was studied in two systems. In the first, the effect of the compounds on the delayed secretion of superoxide anion by adherent neutrophils activated by 1 nM LTB\(_4\) was measured. LY293111 was a very potent inhibitor in this assay (IC\(_{50}\) = 0.5 \(\mu M\)), being more than 1500-fold greater than that for SC-41930 (IC\(_{50}\) = 784 nM). In the other oxidant-producing system, continuously agitated suspended cells were activated with 30 nM LTB\(_4\) or FMLP, and the rate of formation of luminol-dependent chemiluminescence was measured. LY293111 was a very strong antagonist of this reaction (IC\(_{50}\) = 20 ± 2 nM).

![Fig. 2. Inhibition of specific \([^{3}\text{H}]\text{LTB}_4\) binding to intact human neutrophils](image-url)
when the cells were activated with LTB4 (Fig. 4). In contrast, when cells were activated with fMLP, the compound was more than 2 orders of magnitude less inhibitory (IC50 = 4.5 ± 0.5 μM). LY293111 alone at 10 μM did not induce any oxidant production.

Inhibition of Homotypic Aggregation of Human Neutrophils. Both LY293111 and LY255283 inhibited LTB4-induced aggregation of human neutrophils (Fig. 5). However, LY293111 (IC50 = 32 ± 5 nM) was a 4-fold more potent antagonist than LY255283 (IC50 = 125 nM). Neither compound greatly inhibited aggregation induced by fMLP. The IC50 for LY293111 using the peptide as the aggregating agent was 11.0 ± 6.4 μM, whereas the corresponding value for LY255283 was a value greater than 10 μM because the compound inhibited only 30% of the fMLP-induced response at this concentration. At 10 μM or less in concentration, LY293111 did not induce aggregation of neutrophils.

Effects on LTB4-Induced Responses with Guinea Pig Lung and Spleen Membranes and Strips of Parenchyma and Trachea. Before carrying out in vivo studies in guinea pigs with LY293111, experiments were done in vitro to determine whether the compound was as effective at antagonizing LTB4 activation of guinea pig cells as it was at arresting such events with human cells. Figure 6 shows that the compound inhibited very effectively binding of radioactive LTB4 to guinea pig lung membranes. The Kᵢ calculated from this data was 7.1 ± 0.8 nM (n = 40). Thus, the second-generation compound was a 14-fold more potent inhibitor than LY255283 (Kᵢ = 100 nM; Silbaugh et al., 1992). For reference, the corresponding Kᵢ for nonradioactive LTB4 was 0.12 ± 0.01 nM (n = 121). In addition to these studies with lung membranes, the compound also inhibited binding of radiolabeled LTB4 to guinea pig spleen membranes (IC50 = 65 nM; n = 2). LY293111 was also evaluated for its ability to block LTB4-evoked contractions of lung parenchyma strips. At 10 nM, the compound caused a parallel rightward shift of the LTB4 concentration-response curve, suggesting competitive inhibition at this concentration with a pKᵢ value of 8.69 ± 0.16 (Fig. 7). At higher concentrations, however, the curves were shifted in a nonparallel fashion, suggesting that either noncompetitive inhibition or slow dissociation from the receptor was occurring. Silbaugh et al. (1992) reported
that LY255283, when tested in a similar fashion, was a competitive inhibitor with a pA₂ value of 7.17. Thus, in this tissue system, LY293111 is a more potent antagonist than LY255283 by 1 order of magnitude. At 1 μM, the compound had no effect on contractions induced with LTD₄, histamine, carbachol, and the thromboxane A₂ (TxA₂) mimetic agent U46619. Incubation of ovalbumin-sensitized guinea pig tracheal strips with 10 μM LY293111 for 30 min failed to alter the cumulative concentration-response curve obtained on challenging the tissue with increasing concentrations of ovalbumin (Fig. 8). This shows that antagonism of LTB₄ receptors by LY293111 did not influence the antigen-triggered formation of cysteinyl leukotrienes and the subsequent contractions of guinea pig tracheal strips induced by this class of leukotrienes.

Inhibition of Eicosanoid Production. Experiments were conducted to determine whether LY293111 could inhibit the synthesis of either 5-lipoxygenase or cyclooxygenase products. The initial test system investigated was one in which LTB₄ and thromboxane B₂ (TxB₂) were synthesized by plasma-depleted human blood in which the cells were stimulated with 100 nM fMLP and 1 unit/ml thrombin. LY293111 inhibited formation of both eicosanoids. The IC₅₀ for inhibition of LTB₄ synthesis was 1.1 ± 0.5 μM, whereas the corresponding value for preventing TxB₂ formation was 2.4 ± 0.1 μM. We also determined the effect of LY293111 on eicosanoid production in the presence of plasma. Whole blood was stimulated with A23187 alone or in the presence of 150 μM arachidonic acid. At high concentrations of LY293111, a dose-dependent reduction in LTB₄ production occurred whether cells were stimulated only with A23187 (IC₅₀ = 13.7 ± 6.6 μM; n = 3) or both A23187 and arachidonic acid (IC₅₀ = 28.4 ± 15.1 μM; n = 3). In contrast to the results obtained with plasma-depleted blood, no reduction in TxB₂ was observed at concentrations as high as 100 μM arachidonic acid. To further explore the mechanism of inhibition of LTB₄ release, a cytosolic fraction was prepared from human neutrophils, and the effect of LY293111 on LTB₄ formation from arachidonic acid was determined. The compound inhibited at high concentrations. Using substrate concentrations of 1 and 3 μM arachidonic acid, the IC₅₀ values were 0.63 ± 0.016 μM and 0.74 ± 0.11 μM, respectively (n = 3).

Discussion

The main objective of this study was to develop a selective LTB₄ receptor antagonist that was a more potent inhibitor of cell functions activated by the lipid mediator than either of our first-generation compounds, LY223982 and LY255283. The new antagonist, LY293111, was far superior to them at blocking neutrophil functions stimulated by LTB₄. It inhibi-
ited LTB₄-induced chemotaxis more than 950-fold better than the other two compounds and was a 2- to 4-fold better inhibitor of LTB₄-induced neutrophil aggregation. The compound was a very effective inhibitor of oxidant production by LTB₄-activated cells. LY293111 was at least 3 orders of magnitude more potent than LY255283 (Schultz et al., 1991) at preventing prolonged oxidant production by neutrophils that had been allowed to adhere and spread out onto a plastic surface. In addition, the new antagonist also strongly inhibited oxidant production when LTB₄ was added to suspended neutrophils. In this test in the absence of an inhibitor a short respiratory burst occurred during the first few minutes of reaction before the cells have had an opportunity to adhere to any kind of a surface.

Specificity studies indicated that LY293111 was also very selective. The IC₅₀ values for inhibiting both respiratory burst and aggregation of human neutrophils induced by fMLP were more than 2 orders of magnitude higher than corresponding values from LTB₄-induced responses.

LTB₄ receptors on human neutrophils exist in both high and low affinity states (Goldman and Goetzl, 1984). Chemotaxis, adherence, and aggregation are functions mediated by activation of high affinity receptors; degranulation and superoxide production are expressed on stimulation of low affinity receptors. The inhibition of both chemotaxis and the respiratory burst of suspended cells induced with LTB₄ by LY293111 indicated that the compound bound to both receptor states.

LY293111 inhibited LTB₄-stimulated contraction of lung parenchyma strips from guinea pigs. The nature of this inhibition, however, varied with concentration. At 10 nM, LY293111 caused a parallel displacement of the agonist concentration-response curve to the right with no depression of the maximal response. These results are consistent with reversible competitive binding of LY293111 and LTb₄ at the same receptor. At higher concentrations, a nonparallel shift of the agonist concentration-response plot with a concomitant lowering of the maximal response was observed, suggesting either reversible noncompetitive or irreversible inhibition. LTB₄ contracts parenchyma by an indirect mechanism dependent on formation of myotropic cyclooxygenase products, chiefly TxA₂. Indomethacin and other cyclooxygenase inhibitors block the reaction (Sirois et al., 1985). At high micromolar concentrations, LY293111 inhibited synthesis of TxA₂ in experiments where plasma-depleted human blood was stimulated with thrombin (IC₅₀ = 2.4 μM). This raised the question of whether LY293111 inhibited the LTB₄-induced parenchyma contractions not by binding to the LTB₄ receptor but by preventing the synthesis or binding of TxA₂ at its receptor. This seems unlikely. The concentrations of LY293111 tested on the parenchyma were 10 to 300 nM. These amounts would have been too low to have inhibited much thromboxane formation. In addition, at 1 μM, LY293111 did not inhibit contractions caused by LTD₄. Approximately 40 to 50% of the response induced by this eicosanoid is also due to the formation of TxA₂ (Sirois et al., 1985). The compound also at 1 μM did not inhibit contractions induced by the thromboxane mimetic U46619, indicating that LY293111 did not bind to the thromboxane receptor. A possible explanation for the noncompetitive inhibition of LTB₄-induced contractions seen at LY293111 concentrations higher than 10 nM could be a very strong bonding of the compound to the guinea pig LTB₄ receptor. Spaethe and Froelich (unpublished observations) found that isolated human neutrophils exposed to 0.3 to 1.0 μM LY293111 and then extensively washed could no longer bind [³H]LTB₄ at the LTB₄ receptor. A similar tight binding of the compound to guinea pig receptors at the higher nanomolar concentrations may be occurring in the lung parenchyma strip.

Although LY223982 and LY293111 had similar IC₅₀ values for inhibition of LTB₄ binding, the latter compound was a considerably stronger inhibitor of LTb₄-induced chemotaxis. Similar dissociation of potencies in different assays have been observed for other LTB₄ receptor antagonists. There are several possible reasons for these results. Perhaps LY293111 bound better to the high affinity receptors involved in the chemotactic process, whereas LY223982 bound preferentially to the low affinity receptor. A second possible reason might be that LY293111 has LTB₄-specific inhibitory activities on chemotaxis other than just that of antagonizing the LTB₄ receptor, whereas LY223982 does not. Finally, the chemotaxis assay was conducted in a buffer containing 0.05% human serum albumin, whereas [³H]LTb₄ binding studies were carried out in an environment of 0.1% ovalbumin. The two compounds may have very different binding characteristics to serum albumin, and this may account for the differences in their activity in the chemotaxis assay.

At micromolar concentrations, LY293111 blocked synthesis of eicosanoid products. When plasma-depleted human blood was stimulated with FMLP and thrombin, both syntheses of LTB₄ and Tx₄ were inhibited by LY293111. In contrast, when cells in whole blood were activated with A23187, only LTb₄ production was inhibited. Further studies are needed to determine how eicosanoid formation is being inhibited. At these high concentrations, the compound inhibited formation of LTB₄ on incubation of human neutrophil cytosol with arachidonic acid, but this could have been a nonspecific cation chelating effect on LTB₄-forming enzymes. The major action of the compound is that of selectively antagonizing the LTB₄ receptor at nanomolar concentrations.

Several other potent second-generation LTB₄ receptor antagonists have been disclosed in recent years. Shown in Table 1 are the reported activities of these compounds as well as LY293111 at inhibiting LTB₄ binding to intact human neutrophils and preventing activation of polymorphonuclear leukocyte cell functions by the dihydroxy leukotriene. In addition, the effectiveness of each compound when administered orally to abrogate in vivo phenomena induced by LTB₄ is also given. Comparisons of this nature are not easy to make because different laboratories do not necessarily carry out a particular assay or in vivo study under identical conditions. Nevertheless, some general conclusions can be drawn. All of the other compounds were better antagonists of LTB₄ binding than LY293111. Some, such as SC-53228 and SB-209247, were more than 10-fold greater in potency. On the other hand, in LTB₄-activated cell assays, LY293111 was in many cases either equipotent or considerably more inhibitory. The one exception was CGS-25019C, which in most in vitro cell tests was a more potent inhibitor than LY293111. These results again show the lack of correlation between inhibition of receptor binding and receptor activation, suggesting that some of these compounds may have regulatory effects on receptor activation. The effect, however, is apparently selective for LTB₄-activated cellular events because, where inves-
tigated, these compounds either inhibited very weakly or not at all cellular events induced by other agonists. It is somewhat more difficult to draw generalizations from comparisons of in vivo results because of differences in species, type of test, and presumed variations in the absorption, metabolism, and excretion of each compound. Nevertheless, LY293111 and SC-53228 appear to be more potent at inhibiting LTB₄-stimulated responses in vivo than the other compounds.

In addition to the cell responses described herein that are selectively inhibited by LY293111, Marder et al. (1995) reported that the compound also selectively blocked LTB₄-induced calcium mobilization and up-regulation of CD11b/CD18 on isolated human neutrophils. The latter finding has been used to develop an ex vivo assay for measuring the pharmacodynamics of the compound on dosing subjects orally (Marder et al., 1996). Efficacy results with this test, which is based on incubating samples of venous blood with 10 nM LTB₄ and measuring the amount of CD11b up-regulation by flow cytometry, have been shown to correlate well with immunohistochemical analyses of skin biopsies (van Pelt et al., 1997). The ease of operation of this assay is allowing clinical studies to be carried out to determine the usefulness of LY293111 as a therapeutic agent for treatment of inflammatory disorders.

In conclusion, these in vitro studies reveal that LY293111 is a selective LTB₄ receptor antagonist with greatly improved potency in cell function assays over first-generation antagonists. Coupled with results showing potent activity in vivo on oral administration (S. M. Silbaugh and P. W. Stengel, unpublished observations), this agent should prove useful in defining the role of LTB₄ in inflammatory diseases.

**References**


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