0026-895X/92/050873-07$03.00/0

MOLECULAR PHARMACOLOGY, 41:873-879

Characterization of a 5-Lipoxygenase-Activating Protein Binding Assay: Correlation of Affinity for 5-Lipoxygenase-Activating Protein with Leukotriene Synthesis Inhibition

STELLA CHARLESON, PETPIBOON PRASIT, SERGE LÉGER, JOHN W. GILLARD, PHILIP J. VICKERS, JOSEPH A. MANCINI, PETER CHARLESON, JOCELYNE GUAY, ANTHONY W. FORD-HUTCHINSON, and JILLY F. EVANS

Departments of Pharmacology (S.C., P.J.V., J.A.M., A.W.F.-H., J.F.E.), Medicinal Chemistry (P.P., S.L., J.W.G.), Radiopharmaceuticals (P.C.), and Biochemistry (J.G.), Merck Frosst Centre for Therapeutic Research, Quebec H9R 4P8 Canada

Received September 27, 1991; Accepted January 30, 1992

SUMMARY

A binding assay has been developed to measure the affinity of leukotriene synthesis inhibitors for 5-lipoxygenase-activating protein (FLAP), using human leukocyte membranes as the source of FLAP and a radioiodinated leukotriene synthesis inhibitor, ^{125}I-L-691,831, as ligand. Linearity of specific binding of radiolabeled ligand was demonstrated with increasing protein and ligand concentrations. Saturation analysis of radioligand binding showed a K_d of 6 nM and a B_{max} that, depending on the membrane preparation, varied between 8 and 53 pmol/mg of protein. An excellent correlation was shown between affinity for FLAP in the binding assay and inhibition of leukotriene synthesis in human polymorphonuclear leukocytes for compounds from two structurally distinct classes, namely indoles and quinolines. A large number of membrane-active compounds did not compete with ^{125}I-L-691,831 binding to FLAP. In addition, direct 5-lipoxygenase inhibitors and a selection of eicosanoids were unable to compete for FLAP binding. This study validates a selective binding assay for leukotriene synthesis inhibitors whose protein target is FLAP.

Leukotrienes are derived from arachidonic acid via a two-step reaction (oxygenation followed by dehydration) that is catalyzed by 5-lipoxygenase and yields the unstable epoxide LTA_4, which is enzymatically converted to LTB_4, LTC_4, LTD_4, and LTE_4 (1–3). Due to the potent biological properties of the leukotrienes, a great deal of research has been carried out in the past decade to understand the regulation of leukotriene synthesis and to block the action, or prevent the synthesis, of leukotrienes (1–5). Excellent progress towards novel therapy for human asthma has been made in the area of peptidoleukotriene receptor antagonists (6–8). Direct inhibitors of 5-lipoxygenase have shown some efficacy against asthma in human trials, and such inhibitors have the added potential therapeutic target of inflammatory diseases, such as inflammatory bowel disease (9).

A class of selective leukotriene synthesis inhibitors, exemplified by MK-886 (see Fig. 1), has been characterized and developed for the potential treatment of asthma and inflammatory diseases (10). MK-886 has been shown to partially block increased urinary LTE_4 excretion associated with antigen challenge in asthmatics and to partially inhibit antigen-induced bronchoconstriction (11). MK-886 was shown not to inhibit 5-lipoxygenase directly but, rather, to act on a membrane-bound protein termed FLAP (12). FLAP selectively bound a photoaffinity analog of MK-886 and also bound to affinity gels based on MK-886 (12). FLAP was purified from rat neutrophils, and partial amino acid sequences were obtained from several regions of the protein (12). Using oligonucleotide probes complementary to the rat amino acid sequences, full-length cDNAs corresponding to rat and human FLAP were obtained (13). The cDNAs code for proteins of 161 amino acids, which have 92% species homology between rat and human but no strong identity to any other known protein (13).

Expression of 5-lipoxygenase and FLAP in cells correlates

ABBREVIATIONS: LTA_4, 5,6-oxido-7,9,11,14-eicosatetraenoic acid; LTB_4, (5*S*,12*R*)-dihydroxy-6,14-(*cis*)-8,10-(*trans*)-eicosatetraenoic acid; LTC_4, (5*S*)-hydroxy-(6*R*)-*S*-glutathionyl-7,9-(*trans*)-11,14-(*cis*)-eicosatetraenoic acid; LTD_4, (5*S*)-hydroxy-(6*R*)-*S*-cysteinylglycyl-7,9-(*trans*)-11,14-(*cis*)-eicosatetraenoic acid; LTE_4, (5*S*)-hydroxy-(6*R*)-*S*-cysteinyl-7,9-(*trans*)-11,14-(*cis*)-eicosatetraenoic acid; (5*S*)-HPETE, (5*S*)-hydroperoxy-6-(*trans*)-8,11,14-(*cis*)-eicosatetraenoic acid; (5*S*)-HETE, (5*S*)-hydroxy-6-(*trans*)-8,11,14-(*cis*)-eicosatetraenoic acid; (15*S*)-HETE, (15*S*)-hydroxy-5,8,11-(*cis*)-13-(*trans*)-eicosatetraenoic acid; MK-886, 3-[1-(*p*-chlorobenzyl)-5-isopropyl-3-*tert*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid (formerly designated L-663,536); MK-571, 5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phenyl)-4,6-dithianonanedioate *N,N*-dimethylamide carboxylic acid 2-amino-2-methyl-1-propanoic acid (formerly designated L-660,711); L-656,224, 7-chloro-2-[(4-methoxyphenyl)methyl]-3-methyl-5-propyl-4-benzofuranol; L-670,596, 6,8-difluoro-9-*p*-methylsulfonylbenzyl-1,2,3,4-tetrahydrocarbazo-1-yl-acetic acid; REV 5901, 3-(1-hydroxypentyl)-1-(quinoline-2-ylmethoxy) benzene (designated L-656,323 in Merck terminology); DTT, dithiothreitol; PMN, polymorphonuclear leukocytes; Me_2SO, dimethyl sulfoxide; IC_{50}, 50% inhibitory concentration; FLAP, 5-lipoxygenase-activating protein.

with their ability to produce leukotrienes (14). Co-transfection of 5-lipoxygenase and FLAP cDNAs into osteosarcoma cells demonstrated that expression of both these proteins is necessary for leukotriene synthesis (13). In the human promyelocytic HL-60 cell line, differentiated towards a granulocytic lineage by exposure to Me_2SO, coinduction of 5-lipoxygenase and FLAP occurs concurrent with an increased capacity to synthesize leukotrienes (14). MK-886 inhibits leukotriene synthesis in all these cells (13, 14).

The mechanism by which FLAP facilitates 5-lipoxygenase activity in cells is not yet fully understood. However, it has been shown that, after ionophore challenge of cells, 5-lipoxygenase translocates from the cytosol to membranes before leukotriene synthesis (15, 16). MK-886 blocks and reverses the cellular translocation of 5-lipoxygenase, in contrast to structurally similar compounds that are unable to inhibit leukotriene synthesis (17). The correct orientation of 5-lipoxygenase in the membrane is probably favored by interaction with FLAP.

A second class of leukotriene synthesis inhibitors, based on a quinoline structure, have been shown to compete with indole binding to FLAP (18). The affinity of the quinolines for FLAP correlated well with their ability to inhibit leukotriene synthesis in human PMN (18). Labeling of FLAP with the indole photoaffinity probe ^{125}I-L-669,083 was selectively inhibited by a potent quinoline leukotriene synthesis inhibitor, L-674,573 (see Fig. 1). L-674,573 has been shown to block both calcium ionophore- and formylmethionylleucylphenylalanine-induced translocation of 5-lipoxygenase in HL-60 cells (19).

In order to maximize the FLAP binding of both structural classes of inhibitor described above, we have synthesized hybrid molecules containing an indole nucleus fused to a quinoline moiety, and we have termed these compounds quindoles (20). We synthesized a radioiodinated quindole with a high affinity for FLAP, namely ^{125}I-L-691,831, to use as a noncovalent ligand for a FLAP binding assay. As the source of FLAP we used human leukocyte membranes, in which FLAP was previously characterized by photoaffinity labeling, immunoprecipitation, and immunoblot analysis (14, 18). The FLAP binding assay characterization presented here provides further evidence for the correlation between potency of leukotriene synthesis inhibition and affinity for FLAP, for several classes of leukotriene synthesis inhibitors.

Experimental Procedures

^{125}I-L-691,831 synthesis and purification. ^{125}I-L-691,831 (Fig. 1) was synthesized from the methyl ester of the tin tributyl derivative of L-691,831.[1] A solution of chloramine-T (20 μg) in dimethylformamide (10 μl) was added to a mixture of the methyl ester of the tin tributyl derivative of L-691,831 (11 μg) and Na^{125}I (9 mCi), in dimethylformamide (60 μl). The reaction was agitated for 4 min and then evaporated to dryness. The resulting [^{125}I]iodoester was purified by thin layer chromatography (E. Merck 5554 silica gel; solvent, hexane/ethyl acetate, 2:1) and saponified in 250 μl of methanol/0.5 N aqueous NaOH (1.5:1) overnight at room temperature. The saponification mixture was acidified with citric acid, loaded onto a PRP-1 (Hamilton) cartridge, washed with water, and eluted with methanol. The methanolic solution, containing ^{125}I-L-691,831 (1–2 mCi; 1000 mCi/mmol), was applied to a C_{18} reverse phase high pressure liquid chromatography column (Waters, 3 × 30 mm) eluting at 1.0 ml/min with a methanol/water/acetic acid

[1] P. Prasit, unpublished observations.

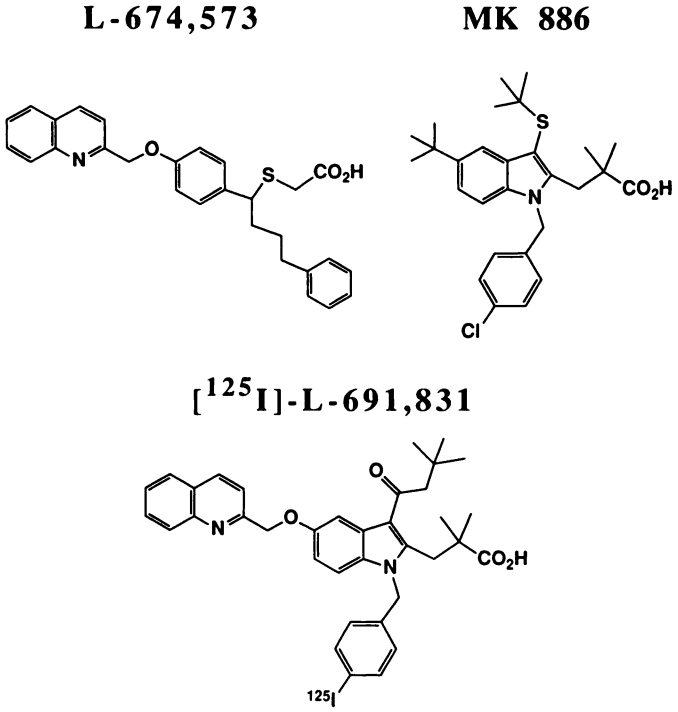

Fig. 1. Structures of leukotriene synthesis inhibitors.

(85:15:0.1) solvent. The pure ^{125}I-L-691,831 eluted at approximately 14 min and was collected, neutralized with NaOH, and dried in a rotary evaporator. The product was redissolved in methanol to approximately 1 μCi/μl (1.5 μCi/pmol) and was stored in aliquots at −70°. The ^{125}I-L-691,831 was chemically stable for up to 4 months when stored under these conditions. Using the same reverse phase chromatographic separation and quantitation, ^{125}I-L-691,831 was stable under the standard binding assay conditions for 20 min at room temperature (data not shown).

Human leukocyte membrane preparation. Human leukocytes were prepared from buffy coat concentrates (courtesy of the Canadian Red Cross, Montreal, Canada) by dextran sedimentation and hypotonic lysis of contaminating red blood cells. Leukocytes were lysed at 2×10^8 cells/ml in 50 mM potassium phosphate buffer, pH 7.1, 0.1 M NaCl, 2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 60 μg/ml soybean trypsin inhibitor, and were subjected to sequential centrifugation at 10,000 × *g* and 100,000 × *g*, for 15 and 60 min (at 4°), respectively. The 100,000 × *g* pellet was resuspended in 20 mM potassium phosphate buffer, pH 7.1, 2 mM EDTA, 1 mM DTT, and was stored in 1-ml aliquots, at approximately 5 mg of protein/ml, at −70° before use.

FLAP binding assay conditions. Aliquots of the human leukocyte membrane preparation were thawed, sonicated briefly, and diluted to 0.05–0.2 mg of protein/ml of assay buffer (0.1 M Tris·HCl, pH 7.5, 0.14 M NaCl, 2 mM EDTA, 0.5 mM DTT, 5% glycerol, 0.05% Tween 20). This diluted membrane suspension was added in 100-μl aliquots (5–20 μg of protein) to 12-mm × 75-mm polypropylene tubes containing 2 μl of Me_2SO or compound in Me_2SO, 100 μl of assay buffer, and 5 μl of ^{125}I-L-691,831 (50–250 pM final concentration) diluted in methanol/assay buffer (1:1). The contents were mixed and incubated at room temperature for 20 min. Incubations were terminated by the addition of 4 ml of ice-cold 0.1 M Tris·HCl, pH 7.5, 0.05% Tween 20 (TT buffer), and immediate filtration through GF-B (Whatman) filters presoaked in TT buffer. Tubes and filters were rinsed with two 4-ml aliquots of ice-cold TT buffer. Filters were transferred to 12-mm × 5.5-mm polystyrene tubes for measurement of bound radioactivity by γ-scintillation counting, in an LKB 1272 Clinigamma Quatro counter. Competition assays were performed in duplicate.

Specific binding is defined as total binding minus nonspecific binding. Total binding is the amount of ^{125}I-L-691,831 bound to membranes

in the absence of competitor. Nonspecific binding is the amount of ^{125}I-L-691,831 bound to membranes that is not displaceable by 10^{-5} M MK-886. For competitive titration curves, the IC_{50} calculation was computed using EBDA software and is defined as the concentration at which there is 50% inhibition of specific ^{125}I-L-691,831 binding.

For association-dissociation experiments, incubations were initiated by addition of 2.2 ml of diluted membrane suspension to stirred bulk mixtures containing 44 μl of Me_2SO or MK-886 (10^{-5} M final concentration in Me_2SO), 2.2 ml of assay buffer, and 110 μl of ^{125}I-L-691,831 (50–250 pM final concentration in methanol/assay buffer, 1:1), all at room temperature. At the specified time intervals, 200-μl aliquots of incubation mixture were removed, added to polypropylene tubes containing 4 ml of ice-cold TT buffer, and filtered immediately through GF-B (Whatman) filters presoaked in TT buffer. Tubes and filters were rinsed with two 4-ml aliquots of cold TT buffer, and membrane-bound radioactivity was determined as described above.

Inhibition of leukotriene synthesis in human PMN. Human PMN were isolated from whole blood that had been anticoagulated with citrate (13 mM) and were purified by dextran sedimentation, followed by hypotonic lysis of contaminating red blood cells. Test compounds or vehicle (1 μl of Me_2SO) were placed in 1.5-ml plastic tubes and 0.5 ml of PMN (5×10^5 cells/ml) were added and incubated at 37° for 2 min before the addition of calcium ionophore A23817 (final concentration, 10 μM). Reactions were continued for 5 min at 37° and then terminated by addition of methanol (250 μl). After centrifugation to remove precipitated proteins, 25 μl of the methanolic extract were removed for LTB_4 radioimmunoassay (21). LTB_4 in samples treated with compound was compared with that in control samples (20–30 ng/10^6 PMN), and the IC_{50} values for each compound were estimated from inhibition curves based on five titration points.

FLAP immunoblot analysis. Immunoblot of FLAP protein was performed using a rabbit polyclonal peptide antibody termed H5 (raised to the thyroglobulin conjugate of amino acids 41–52 of FLAP) and ^{125}I-Protein A detection, as described previously (14).

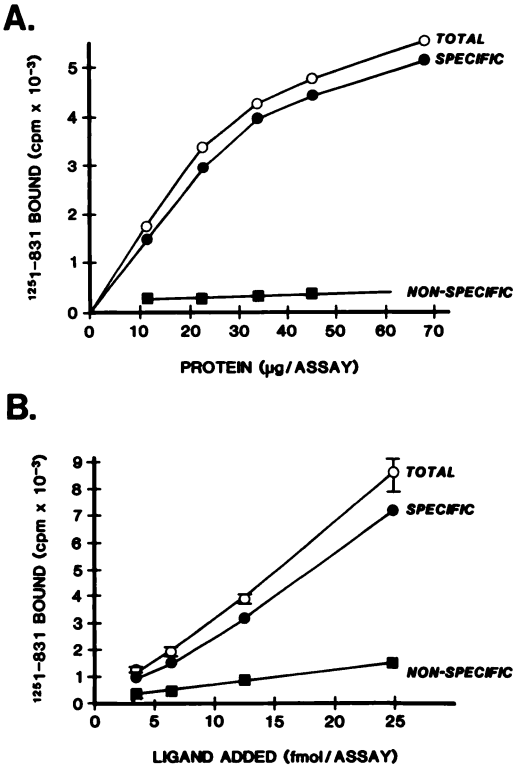

Fig. 2. Binding of ^{125}I-L-691,831 to human leukocyte membranes. A, Linearity of binding with increasing protein concentration. B, Linearity of binding with increasing ligand concentration. Assays were performed as described in Experimental Procedures.

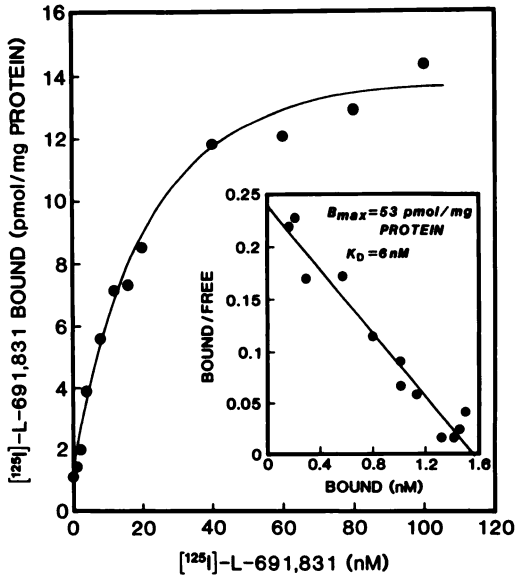

Fig. 3. Saturation analysis of ^{125}I-L-691,831 binding to human leukocyte membranes. Assays were performed as described in Experimental Procedures, using increasing amounts of radiolabeled ligand from 0.1 to 20 pM. Saturation parameters were determined by nonlinear regression analysis of a one-site model fit, using LIGAND software.

FLAP photoaffinity labeling and immunoprecipitation. Photoaffinity labeling of human leukocyte membrane proteins with ^{125}I-L-691,678 (a quindole photoaffinity ligand) (22) and immunoprecipitation with FLAP antiserum H5 were carried out as described previously (14).

Protein determination. Protein concentration was determined by a microtiter plate modification of the Bio-Rad procedure, based on the method of Bradford (23).

Results

The leukotriene synthesis inhibitors L-674,573, MK-886, and L-691,831 are shown in Fig. 1. These are potent representatives of the quinoline, indole, and quindole classes of leukotriene synthesis inhibitors, with IC_{50} values as inhibitors of LTB_4 synthesis in human PMN of 6, 2.2, and 2.5 nM, respectively. Radioiodinated L-691,831 was prepared, as described in Experimental Procedures, and utilized as ligand for FLAP binding studies. Human leukocyte 100,000 × *g* membranes were used as the source of FLAP (12, 14, 18). Under the assay conditions described in Experimental Procedures, specific binding of ^{125}I-L-691,831 increased with respect to increasing amounts of membrane protein (Fig. 2A) and with respect to increasing amounts of ligand (Fig. 2B). Competition binding assays were carried out at protein and ligand concentrations at which linear responses were observed for both variables. Maximum specific binding of ^{125}I-L-691,831 occurred using Tris·HCl, pH 7.5, as buffer (data not shown). Although there was no enhancement of specific binding by the addition of NaCl, EDTA, DTT, or 5% glycerol, they were included in the FLAP binding assay to be consistent with buffers used to purify FLAP (12). Divalent cations were not included in our standard assay, but at 100 mM concentrations magnesium produced a 1.4-fold and calcium a 2.2-fold increase in specific binding (data not shown). The kinetics of association and dissociation of ^{125}I-L-691,831 to human leukocyte membrane protein were very rapid. At room temperature, 50% maximum specific binding occurred in <30 sec, and dissociation produced by excess MK-886 was equally

rapid. There was little difference in either amount of binding or kinetics of binding of ^{125}I-L-691,831 if the incubation was carried out at 4°, compared with room temperature (data not shown).

A full saturation analysis of ^{125}I-L-691,831 binding to human leukocyte membrane protein was performed, and binding parameters determined by nonlinear regression Scatchard analysis were consistent with a one-site model fit, using LIGAND software (Fig. 3). This analysis demonstrated a dissociation constant for ^{125}I-L-691,831 of 6 nM and a maximum number of binding sites of 53 pmol/mg of protein.

A number of different preparations of membranes demonstrated similar dissociation constants for ^{125}I-L-691,831 (5–7 nM), but maximum numbers of binding sites varied from 8 to 53 pmol/mg of protein. Values for maximum binding of ^{125}I-L-691,831 correlated well with the amount of FLAP in the membrane preparations, as determined by both immunoblot analysis (Fig. 4A) and photoaffinity labeling of FLAP (Fig. 4B). Immunoblot analysis was carried out using antibody H5, which was generated to human FLAP amino acid residues 41–52. A comparison was made with two membrane preparations, which varied in maximum binding site capacity for ^{125}I-L-691,831 from 8 (preparation 1) to 53 (preparation 2) pmol/mg of protein,

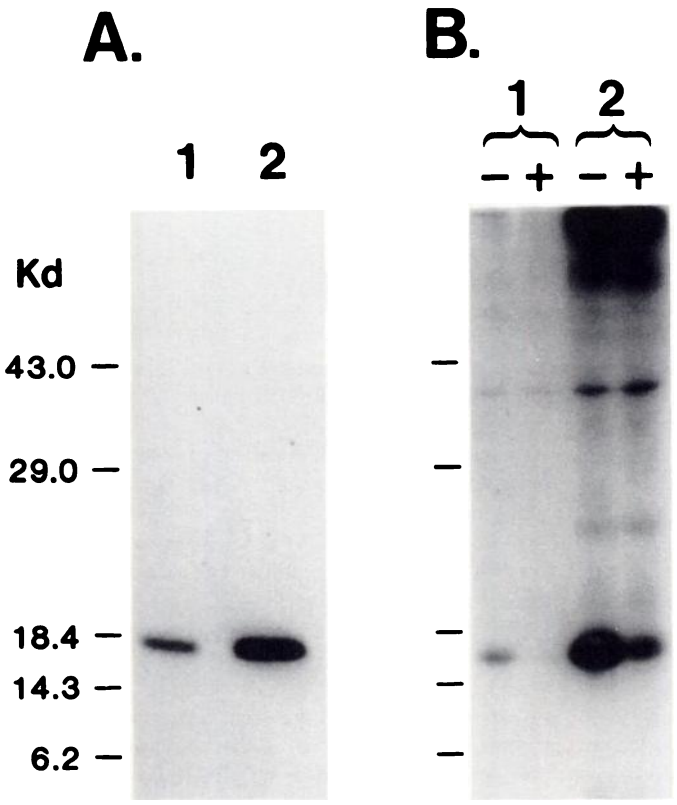

Fig. 4. A, Immunoblot analysis of FLAP. Equal amounts (100 μg) of membrane protein from two different human leukocyte membrane preparations were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 13.5% polyacrylamide gels and were probed with FLAP antibody, as described in Experimental Procedures. B, Photoaffinity labeling of FLAP. Equal amounts (1 mg) of membrane protein from two different human leukocyte membrane preparations were labeled with ^{125}I-L-691,678, in the absence (−) or presence (+) of 1 μM MK-886, and immunoprecipitated with FLAP antibody, and immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography, as described in Experimental Procedures. *1*, Membrane preparation 1; *2*, membrane preparation 2.

i.e., a 6.6-fold increase in preparation 2, compared with preparation 1. This agreed well with quantitation of the FLAP immunoblot and immunoprecipitation analyses, which demonstrated a 5- and 10-fold greater concentration of FLAP, respectively, in preparation 2, compared with preparation 1.

In order to demonstrate the physiological relevance of the FLAP binding assay, the correlation of the binding results with the inhibition of leukotriene synthesis in human PMN was investigated. A strong linear correlation (r = 0.97) was seen between the ability of indoles to specifically compete with ^{125}I-L-691,831 binding to human membrane protein and their potency as leukotriene synthesis inhibitors (Fig. 5). The IC_{50} values for competition of FLAP binding were 10–20 times greater than the IC_{50} values observed in the human PMN leukotriene inhibition assays. The concentration of FLAP in the membrane preparations or the human leukocytes is unknown, but it is probable that the differences in IC_{50} values between the binding assay and the leukotriene inhibition assay are related to different FLAP concentrations in the two assays. It has been previously observed with these compounds that their IC_{50} values for whole-cell leukotriene synthesis inhibition increase with increasing cell number. For example, MK-886 has an IC_{50} of 2.2 nM for inhibition of ionophore-challenged leukotriene production in human leukocytes at 5×10^5 cells/ml (10), but this increases to an IC_{50} of approximately 100 nM at 4×10^7 cells/ml (17).

Further evidence of the interaction of indole leukotriene synthesis inhibitors with a specific protein is shown by the stereoselective competition of the monomethyl isomers of MK-886, namely L-680,305 and L-680,306, as shown in Fig. 6. The IC_{50} values for L-680,305 and L-680,306 were 75 nM and 820 nM, respectively, in the FLAP binding assay (three experiments) and 7 nM and 37 nM, respectively, in the PMN leukotriene synthesis inhibition assay (two experiments).

The correlation of affinity for FLAP in the FLAP binding assay with leukotriene synthesis inhibition, for a quinoline class of leukotriene synthesis inhibitors known to bind to FLAP (18), was investigated. As illustrated in Fig. 7, there was a good linear correlation (r = 0.9) between the ability of the quinoline leukotriene synthesis inhibitors to compete in the FLAP binding assay and their potency for inhibition of PMN leukotriene synthesis.

In order to produce more potent leukotriene synthesis inhibitors, we synthesized hybrid molecules containing an indole nucleus fused to a quinoline moiety, on the left side of the molecule, and we termed these molecules quindoles (20). The ligand itself in our FLAP binding assay, namely ^{125}I-L-691,831, is a quindole. A considerable number of quindoles have been synthesized, and these were all potent leukotriene synthesis inhibitors and showed strong affinity for FLAP in our FLAP binding assay (data not shown). Because compounds in the quindole class were all potent leukotriene synthesis inhibitors, we were unable to make a correlation over a range of concentrations of quindoles, as was the case with the indoles (Fig. 5) and the quinolines (Fig. 7).

As part of our investigation of the ^{125}I-L-691,831 binding site on FLAP, we attempted competition of a large number of compounds, from a wide variety of structural classes, at 10 μM. These included compounds known to act on other membrane protein targets, such as chlorpromazine, timolol, mepyramine, cyproheptadine, and isoproteronol. None of these compounds

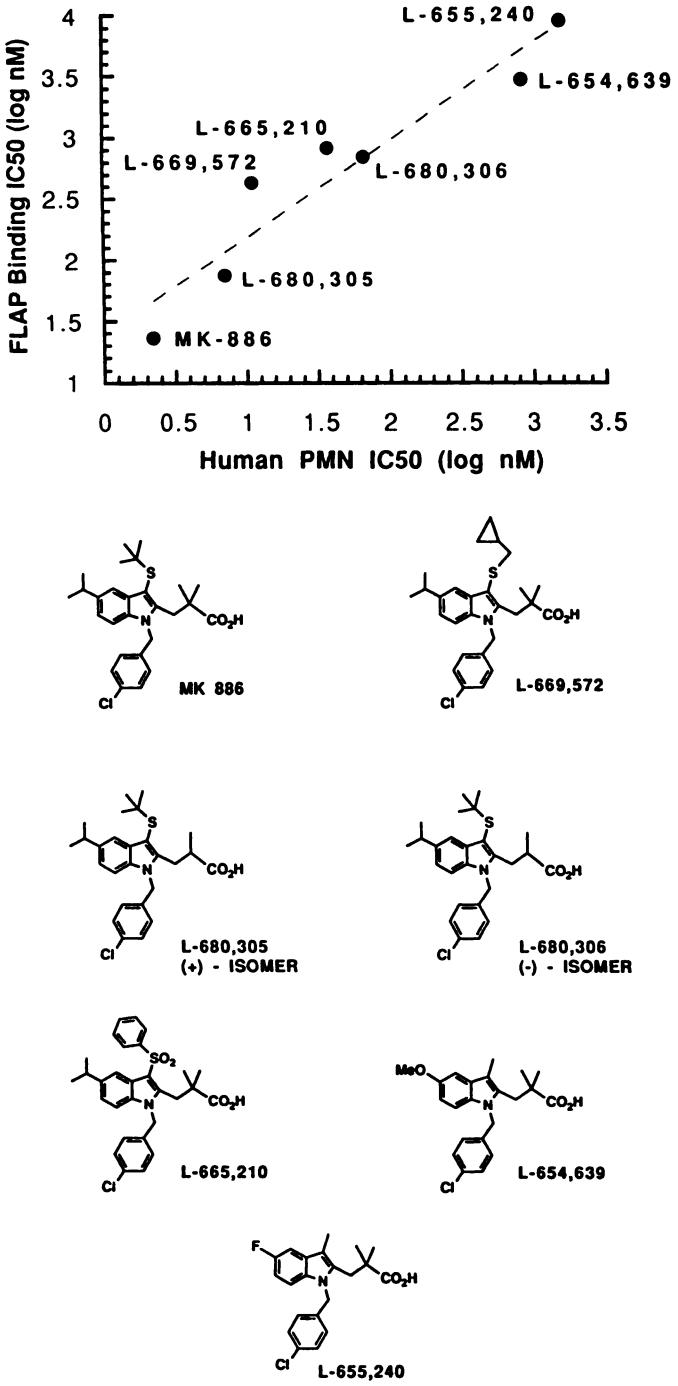

Fig. 5. *Upper*, correlation of competition of ^{125}I-L-691,831 binding to human leukocyte membranes, by indole leukotriene synthesis inhibitors, with leukotriene synthesis inhibition in human PMN. Assays were performed as described in Experimental Procedures. ^{125}I-L-691,831 competition curves were carried out in duplicate at six to eight concentrations of competitor, and values are the means of at least two separate experiments. PMN leukotriene synthesis inhibition titrations were performed with five concentrations of inhibitor, and values represent the average of two to 10 titrations. *Lower*, chemical structures of the compounds used.

at 10 μM affected ^{125}I-L-691,831 binding to human leukocyte membranes (data not shown). Other compounds that might have interacted with FLAP were tested versus ^{125}I-L-691,831 binding to human leukocyte membranes (Table 1). These included arachidonic acid, because we felt that the role of FLAP

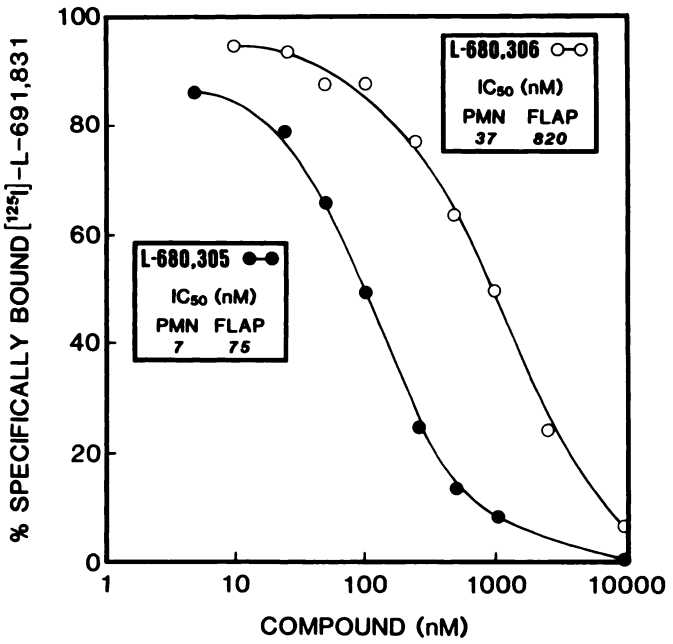

Fig. 6. Stereoselective competition of the monomethyl enantiomers of MK-886, i.e., L-680,305 and L-680,306, for ^{125}I-L-691,831 binding to human leukocyte membranes. Assays were carried out as described in Experimental Procedures, in duplicate, and points are means of duplicate assays. These curves are representative of three titrations.

might be to facilitate the transfer of arachidonic acid from a phospholipase to 5-lipoxygenase. As shown in Table 1, arachidonic acid (20 μM) or its metabolites, (15*S*)-HETE, (5*S*)-HETE, (5*S*)-HPETE, LTB_4, LTC_4, and LTD_4, did not interact with the ^{125}I-L-691,831 binding site on FLAP. The compounds L-656,224, a 5-lipoxygenase inhibitor (24), and L-670,596, a thromboxane antagonist (25), act on proteins that utilize substrates derived from arachidonic acid and may, therefore, have some evolutionary relationship to FLAP. However, these compounds at 10 μM concentration showed no inhibition of ^{125}I-L-691,831 binding to human leukocyte membranes (Table 1). Indomethacin is a cyclooxygenase inhibitor with an indole nucleus, but it also showed no inhibition in our FLAP binding assay (Table 1). Finally, dexamethasone and cromolyn sodium have anti-inflammatory properties, but their complete mechanisms of action have not been elucidated. FLAP might have been a target of these compounds but, as shown in Table 1, these compounds do not inhibit ^{125}I-L-691,831 binding to human leukocyte membranes.

Discussion

This study describes the characterization of a FLAP binding assay. In human leukocyte membranes, a high affinity, saturable binding site for the ligand ^{125}I-L-691,831 has been demonstrated. A correlation has been shown between the ability of a compound to compete with ^{125}I-L-691,831 for binding to FLAP and its ability to inhibit leukotriene synthesis. A correlation has also been shown, with different membrane preparations, between the maximum number of binding sites, for ^{125}I-L-691,831 and the amount of FLAP protein, as quantitated by immunoblot or photoaffinity-labeled immunoprecipitation analyses.

We do not yet know the natural ligand for FLAP. We do not have any evidence for direct interaction of FLAP and 5-lipox-

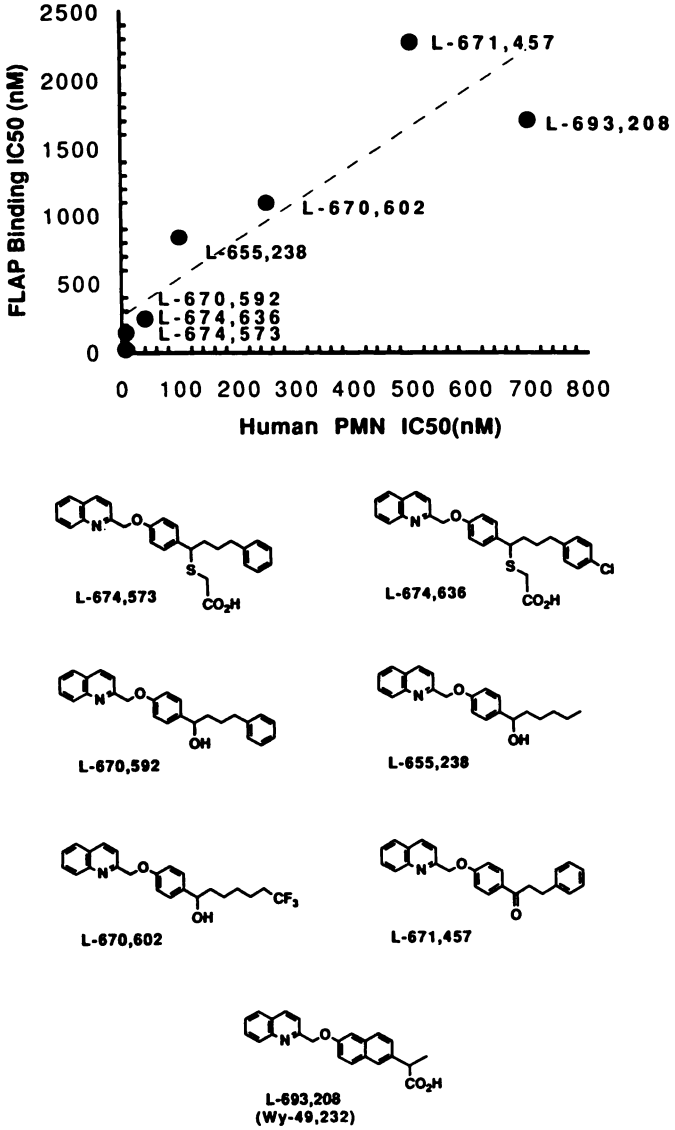

Fig. 7. *Upper*, correlation of competition of ^{125}I-L-691,831 binding to human leukocyte membranes, by quinoline leukotriene synthesis inhibitors, with leukotriene synthesis inhibition in human PMN. Assays were performed as described in Experimental Procedures. ^{125}I-L-691,831 competition curves were carried out in duplicate at six to eight concentrations of competitor, and values are the means of at least two separate experiments. PMN leukotriene synthesis inhibition titrations were performed with five concentrations of inhibitor, and values represent the average of one to six titrations. *Lower*, chemical structures of the compounds.

ygenase. However, if such association occurs, the natural ligand may be a specific epitope of 5-lipoxygenase itself. We have not observed competition of ^{125}I-L-691,831 binding to FLAP by purified 5-lipoxygenase or several 5-lipoxygenase peptide sequences (data not shown). However, activation of 5-lipoxygenase may alter its conformation, and it may be an activated 5-lipoxygenase that is necessary for FLAP recognition. We do not have sufficient quantities of purified FLAP to examine the binding of radioiodinated leukotriene synthesis inhibitors or 5-lipoxygenase. We are attempting to obtain greater quantities of FLAP by recombinant expression in a number of different cell types. We are also currently examining the leukotriene synthesis inhibitor binding site on FLAP by site-specific and deletion mutagenesis studies.

TABLE 1

Selectivity of competition with ^{125}I-L-691,831 binding to human leukocyte membranes

Assays were carried out as described in Experimental Procedures. Results given are means of duplicate determinations, where the range was <20%.

Competitor	IC_{50}
	nM
L-691,831	6
Arachidonic acid	>20,000
(15*S*)-HETE	>20,000
(5*S*)-HETE	>15,000
(5*S*)-HPETE	>15,000
LTB_4	>3,400
LTC_4	>2,900
LTD_4	>3,000
L-656,224	>10,000
L-670,596	>10,000
MK-571	>10,000
Indomethacin	>10,000
Dexamethasone	>10,000
Cromolyn sodium	>10,000

The original quinoline leukotriene synthesis inhibitors, such as REV 5901, were hypothesized to be mimicking (15*S*)-HETE, which may have natural anti-inflammatory properties (26). In our assay, no significant affinity for the ligand binding site was shown by (15*S*)-HETE, (5*S*)-HETE, arachidonic acid, or a selection of leukotrienes (Table 1). REV 5901 had IC_{50} values of 2 μM and 1.8 μM in the FLAP binding and PMN assays, respectively. Because REV 5901 is known to have some direct 5-lipoxygenase inhibitory activity, we have not included it in our FLAP binding versus leukotriene synthesis inhibition in PMN correlation. Only compounds that were relatively inactive versus 5-lipoxygenase activity in a broken cell 5-lipoxygenase assay[2] have been included in our correlation analyses of FLAP binding and PMN leukotriene synthesis inhibition.

We have recently described a potent radioiodinated photoaffinity ligand, namely ^{125}I-L-691,678, based on the hybrid quinoline-indole structure (20, 22). This ligand has been shown to interact directly with human leukocyte FLAP, and competition studies with this ligand also suggest that the quinoline, indole, and quindole classes of compound share a common binding site on FLAP (22).

Although it is clear that FLAP is essential for the cellular activation of 5-lipoxygenase and that the FLAP-binding compounds such as MK-886 inhibit 5-lipoxygenase translocation, much remains to be understood about both the activation and the inhibition of cellular leukotriene synthesis. An important feature of cellular 5-lipoxygenase activation is that the ratio of leukotriene product to (5*S*)-HPETE/(5*S*)-HETE product is 5–10 to 1, compared with an inverse relationship in broken cell 5-lipoxygenase assays. The way in which the association of 5-lipoxygenase with membranes and/or FLAP results in enhancement of the second step in the 5-lipoxygenase reaction, namely dehydration of (5*S*)HPETE to LTA_4, remains to be elucidated. Specific inhibitors of different structural classes, high expression recombinant 5-lipoxygenase systems (27), and antibodies to 5-lipoxygenase and FLAP should allow these questions to be answered.

Acknowledgments

The authors would like to thank D. E. MacIntyre, T. J. Bach, S. Luell, R. Meurer, A. Maycock, D. DeSousa, and D. Nathaniel for PMN leukotriene

[2] D. Riendeau, unpublished observations.

synthesis inhibition assays; D. Riendeau, J. P. Falgueyret, D. Denis, and L. Choo for unpublished 5-lipoxygenase assay data; H. Morton, R. Fortin, R. Zamboni, M. Thérien, and D. Delorme for synthesis of compounds; and D. K. Miller and J. Hutchinson for stimulating discussions and helpful suggestions.

References

1. Samuelsson, B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science (Washington D. C.)* **220**:568–575 (1983).
2. Ford-Hutchinson, A. W., M. A. Bray, M. V. Doig, M. E. Shipley, and M. J. H. Smith. Leukotriene B_4, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature (Lond.)* **286**:264–265 (1980).
3. Dahlen, S. E., P. Hedqvist, S. Hammarstrom, and B. Samuelsson. Leukotrienes are potent constrictors of human bronchi. *Nature (Lond.)* **288**:484–486 (1980).
4. Ford-Hutchinson, A. W. Evidence for the involvement of leukotrienes and other lipoxygenase products in disease states, in *Leukotrienes and Lipoxygenases* (J. Rokach, ed.). Elsevier Science Publishing, New York, 405–425 (1989).
5. Fitzsimmons, B. J., and J. Rokach. Enzyme inhibitors and leukotriene receptor antagonists, in *Leukotrienes and Lipoxygenases* (J. Rokach, ed.). Elsevier Science Publishing, New York, 427–502 (1989).
6. Jones, T. R., R. Zamboni, M. Belley, E. Champion, L. Charette, A. W. Ford-Hutchinson, R. Frenette, J. Y. Gauthier, S. Leger, P. Masson, C. S. McFarlane, H. Piechuta, J. Rokach, H. Williams, R. N. Young, R. N. DeHaven, and S. S. Pong. Pharmacology of L-660,711 (MK-571): a novel, potent and selective leukotriene D_4 receptor antagonist. *Can. J. Physiol. Pharmacol.* **67**:17–28 (1989).
7. Gaddy, J., R. K. Bush, D. Margolskee, V. C. Williams, and W. Busse. The effects of leukotriene D_4 (LTD_4) antagonist (MK-571) in mild to moderate asthma. *J. Allergy Clin. Immunol.* **85**:A197 (1990).
8. Taylor, I. K., K. M. O'Shaughnessy, R. W. Fuller, and C. T. Dollery. Effect of cysteinyl-leukotriene receptor antagonist ICI 201,219 on allergen-induced bronchoconstriction and airway hyperreactivity in atopic subjects. *Lancet* **337**:690–693 (1991).
9. Laursen, L. S., J. Naesdal, K. Bukhave, K. Lauristen, and J. Rask-Madsen. Selective 5-lipoxygenase inhibition in ulcerative colitis. *Lancet* **335**:683–685 (1990).
10. Gillard, J. W., A. W. Ford-Hutchinson, C. Chan, S. Charleson, A. Foster, R. Fortin, S. Leger, C. S. McFarlane, H. Piechuta, D. Riendeau, C. A. Rouzer, J. Rokach, R. Young, E. MacIntyre, L. Peterson, G. Eirman, S. Hopple, K. Hupe, R. Meurer, E. Opas, and S. Pacholok. L-663,536 (MK-886): a novel, orally active leukotriene biosynthesis inhibitor. *Can. J. Physiol. Pharmacol.* **67**:456–464 (1989).
11. Bel, E. H., W. Tanaka, R. Spector, B. Friedman, H. von de Veer, J. H. Diijkman, and P. J. Sterk. MK-886, an effective oral leukotriene biosynthesis inhibitor on antigen-induced early and late asthmatic reactions in man. *Annu. Rev. Respir. Dis.* **141**:A31 (1990).
12. Miller, D. K., J. W. Gillard, P. J. Vickers, S. Sadowski, C. Léveillé, J. A. Mancini, P. Charleson, R. A. F. Dixon, A. W. Ford-Hutchinson, R. Fortin, J. Y. Gauthier, J. Rodkey, R. Rosen, C. Rouzer, I. S. Sigal, C. D. Strader, and J. F. Evans. Identification and isolation of a membrane protein necessary for leukotriene production. *Nature (Lond.)* **343**:278–281 (1990).
13. Dixon, R. A. F., R. E. Diehl, E. Opas, E. Rands, P. J. Vickers, J. F. Evans, J. W. Gillard, and D. K. Miller. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature (Lond.)* **343**:282–284 (1990).
14. Reid, G. K., S. Kargman, P. J. Vickers, J. A. Mancini, C. Léveillé, D. Ethier, D. K. Miller, J. W. Gillard, R. A. F. Dixon, and J. F. Evans. Correlation between expression of 5-lipoxygenase-activating protein, 5-lipoxygenase and cellular leukotriene synthesis. *J. Biol. Chem.* **265**:19818–19823 (1990).
15. Rouzer, C. A., and S. Kargman. Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J. Biol. Chem.* **263**:10980–10988 (1988).
16. Wong, A., S. M. Hwang, M. N. Cook, G. K. Hogaboom, and S. T. Crooke. Interactions of 5-lipoxygenase with membranes: studies on the association of soluble enzyme with membranes and alterations in enzyme activity. *Biochemistry* **27**:6763–6769 (1988).
17. Rouzer, C. A., A. W. Ford-Hutchinson, H. E. Morton, and J. W. Gillard. MK-886, a potent and specific leukotriene biosynthesis inhibitor, blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. *J. Biol. Chem.* **265**:1436–1442 (1990).
18. Evans, J. F., C. Léveillé, J. A. Mancini, P. Prasit, M. Thérien, R. Zamboni, J. Y. Gauthier, R. Fortin, P. Charleson, D. E. MacIntyre, S. Luell, T. J. Bach, R. Meurer, J. Guay, P. J. Vickers, C. A. Rouzer, J. W. Gillard, and D. K. Miller. 5-Lipoxygenase-activating protein is the target of a quinoline class of leukotriene synthesis inhibitors. *Mol. Pharmacol.* **40**:22–27 (1991).
19. Kargman, S., P. Prasit, and J. F. Evans. Translocation of HL-60 5-lipoxygenase: inhibition of A23187- or fMLP-induced translocation by indole and quinoline leukotriene synthesis inhibitors. *J. Biol. Chem.* **266**:23745–23752 (1991).
20. Prasit, P., R. Fortin, J. H. Hutchinson, M. L. Belley, S. Léger, J. W. Gillard, and R. Frenette. (2-Quinolinylmethoxy)indoles as inhibitors of the biosynthesis of leukotrienes. *Canadian Patent Application* 2,023,340 (1991).
21. Rokach, J., E. C. Hayes, Y. Girard, D. L. Lombardo, A. L. Maycock, A. S. Rosenthal, R. N. Young, R. Zamboni, and H. J. Zweerink. The development of sensitive and specific radioimmunoassays for leukotrienes. *Prostaglandins Leukotrienes Med.* **13**:21–25 (1984).
22. Mancini, J. A., P. Prasit, M. G. Coppolino, P. Charleson, S. Léger, J. F. Evans, J. W. Gillard, and P. J. Vickers. 5-Lipoxygenase-activating protein is the target of a novel hybrid of two classes of leukotriene biosynthesis inhibitors. *Mol. Pharmacol.* **41**:267–272 (1992).
23. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
24. Atkinson, J. G., Y. Guindon, and C. K. Lau. Lipoxygenase inhibitors. *European Patent Application* EP 146,243 (1985).
25. Ford-Hutchinson, A. W., Y. Girard, A. Lord, T. R. Jones, M. Cirino, J. F. Evans, J. Gillard, P. Hamel, C. Léveillé, P. Masson, and R. Young. The pharmacology of L-670,596, a potent and selective thromboxane/prostaglandin endoperoxide receptor antagonist. *Can. J. Physiol. Pharmacol.* **67**:989–993 (1989).
26. Musser, J. H., U. R. Chakraborty, S. Sciotino, R. J. Gordon, A. Khandwala, E. S. Neiss, T. P. Pruss, R. Van Inwegan, I. Weinryb, and S. M. Coutts. Substituted arylmethyl phenyl ethers. 1. A novel series of 5-lipoxygenase inhibitors and antagonists. *J. Med. Chem.* **30**:96–104 (1986).
27. Denis, D., J. P. Falgueyret, D. Riendeau, and M. Abramovitz. Characterization of the activity of purified recombinant human 5-lipoxygenase in the absence and presence of leukocyte factors. *J. Biol. Chem.* **266**:5072–5079 (1991).

Send reprint requests to: Jilly F. Evans, Department of Pharmacology, Merck Frosst Centre for Therapeutic Research, P. O. Box 1005, Pointe Claire-Dorval, Quebec H9R 4P8, Canada.