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PHARMACOLOGY OF THE PYRROLOIMIDAZOLE, SK&F 105809—I

INHIBITION OF INFLAMMATORY CYTOKINE PRODUCTION AND OF 5-LIPOXYGENASE- AND CYCLOOXYGENASE-MEDIATED METABOLISM OF ARACHIDONIC ACID

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Abstract—SK&F 105809 {2-(4-methylsulfinylphenyl)-3-(4-pyridyl)-6,7-dihydro-[5*H*]-pyrrolo[1,2-*a*] imidazole} was determined to be a prodrug for the sulfide metabolite SK&F 105561 {2-(4-methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5*H*]-pyrrolo[1,2-*a*] imidazole} which inhibited interleukin-1 (IL-1) production *in vitro* and both 5-lipoxygenase (5-LO) and prostaglandin H (PGH) synthase activities *in vitro* and *ex vivo*. SK&F 105561 inhibited partially purified 5-LO with a half-maximal concentration (IC_{50}) of 3 μM. This inhibition was reversible, independent of preincubation time, and dependent on the concentration of the substrate arachidonic acid. SK&F 105561 also inhibited purified PGH synthase with the potency dependent on the level of peroxidase activity. The IC_{50} was 100 μM in the absence of peroxidase activity, whereas an IC_{50} of 3 μM was observed in the presence of peroxidase activity. Using human monocytes, SK&F 105561 inhibited A23187-stimulated prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) production with IC_{50} values of 0.1 and 2 μM, respectively. In addition, IL-1 production by lipopolysaccharide-stimulated human monocytes was also inhibited (IC_{50} 2 μM). Oral administration of SK&F 105809 to rats resulted in a dose-related generation of SK&F 105561 and in the inhibition of thromboxane B_2 and LTB_4 production *ex vivo* with a half-maximal dose (ED_{50}) of 15 and 60 mg/kg, respectively. SK&F 105561 showed weak inhibitory activity on 12-lipoxygenase with an IC_{50} of greater than 200 μM. Neither SK&F 105561 nor SK&F 105809 inhibited the stimulated-turnover of arachidonic acid-containing phospholipids in human monocytes or the activity of cell-free phospholipases A_2 and C. Moreover, neither SK&F 105561 nor SK&F 105809 antagonized the binding of LTB_4 or leukotriene D_4 to membrane receptors. From these results, SK&F 105561, the active principle of SK&F 105809, acts as an inhibitor of both inflammatory cytokine and eicosanoid production.

The effectiveness of corticosteroids as therapeutic agents for inflammation is well-appreciated, but adverse effects limit their utility [1, 2]. Although the mechanism by which steroids exert their actions is unknown, part of their mechanism is attributed to inhibition of inflammatory cytokine production [1] and a blockade of eicosanoid production via a reduction in substrate availability [3, 4]. Indeed, the clinical manifestations of inflammation that steroids are most capable of reversing (e.g. pain, swelling and cell infiltration) are those actions that are associated with interleukin-1 (IL-1**), tumor necrosis factor (TNF) [5], prostaglandin (PG) and leukotriene (LT) activity [6, 7].

The role of prostanoids as proinflammatory agents and the therapeutic value of inhibiting their synthesis are well-documented [8]. The pathophysiological role of LT metabolites has been the focus of more recent studies [9]. The potent chemotactic and algesic activities of leukotriene B_4 (LTB_4) [10, 11], together with the observation that leukotriene C_4 (LTC_4) and leukotriene D_4 (LTD_4) increase vascular permeability [12–14], have implicated LTs as proinflammatory mediators and their blockade as a target for pharmacological intervention. Clinical suggestions for the participation of 5-lipoxygenase (5-LO) products in inflammatory diseases have also been reported. LTB_4 was detected in rheumatoid and gouty arthritic joint fluid [15, 16], inflammed gastrointestinal mucosa [17], and psoriatic skin [18]. The cysteinyl LTs have been observed in bronchial lavage fluid [19] and arterial blood [20] after immunological challenge. Selective drugs that inhibit

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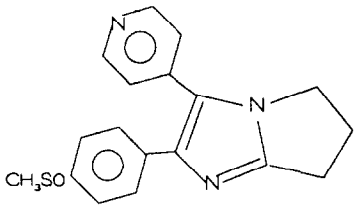
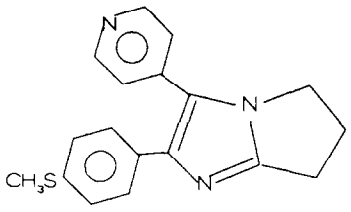
** Abbreviations: IL-1, interleukin-1; ETYA, eicosatetraynoic acid; DHETE, dihydroxyeicosatetranoic acid; HETE, hydroxyeicosatetranoic acid; HPETE, hydroperoxyiecosatetranoic acid; HHT, hydroxyheptadecatrienoic acid; K_m, half-maximal requirement; IC_{50}, half-maximal inhibitory concentration; ID_{50}, half-maximal inhibitory dose; LO, lipoxygenase; LT, leukotriene; NGDA, nordihydroguaiaretic acid; PG, prostaglandin; TNF, tumor necrosis factor; and TxB_2, thromboxane B_2.



SK&F 105809



SK&F 105561

Fig. 1. Structures of SK&F 105809 and SK&F 105561.

either the production or the activity of the LTs are now becoming available so that the role of the LTs in disease can be better understood. Likewise, IL-1 and TNF have been implicated in inflammatory diseases, including rheumatoid arthritis [21, 22].

Eicosanoid production can be inhibited at several steps, which include receptor antagonists, prevention of the signal transduction events following receptor occupancy, inhibition of arachidonic acid release, or inhibition of fatty acid oxygenase activity. Presently, most of our understanding of eicosanoid biosynthesis centers on the fatty acid oxygenases, in particular prostaglandin H (PGH) synthase and 5-LO. Although there are many differences, both PGH synthase and 5-LO belong to the family of dioxygenase enzymes which catalyze a peroxide-dependent oxidation of arachidonic acid.

Because of the similarity in general catalytic mechanism and the shared requirement for arachidonic acid, it is not surprising that there are a number of compounds that are capable of inhibiting both enzymes. Unfortunately, these compounds either fail to inhibit *in vivo* [23] or are far more selective for one enzyme versus the other enzyme [24]. We have identified a compound (SK&F 105561) which inhibits inflammatory cytokine production, PGH synthase and 5-LO and demonstrated anti-inflammatory properties in animal models *in vivo* [25]. This report describes the actions of the prodrug SK&F 105809 and its metabolite SK&F 105561 (Fig. 1) on IL-1 production *in vitro* and on eicosanoid production *in vitro* and *ex vivo*. A preliminary report of some of these data has been presented [26].

MATERIALS AND METHODS

Materials. SK&F 105561 {2-(4-methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5*H*]-pyrrolo[1,2-*a*]imidazole}, SK&F 105809 {2-(4-methylsulfinylphenyl)-3-(4-pyridyl)-6,7-dihydro-[5*H*]-pyrrolo[1,2-*a*]imidazole} (Fig. 1), AA-861 and A63162 were obtained from the Drug Substances and Products Registry of SmithKline Beecham Pharmaceuticals. Eicosanoid standards were purchased from Biomol (Plymouth Meeting, PA). Unless otherwise indicated, all other reagents were obtained from the Sigma Chemical Co. (St. Louis, MO). The indicated concentrations of compounds represent the final concentrations in the assay buffer. Protein concentrations were determined using the BCA protein reagent kit (Pierce, Rockford, IL). Lewis male rats (250 g) were obtained from the Charles River Breeding Laboratories (Kingston, NY).

5-Lipoxygenase activity (5-LO). 5-LO was isolated from RBL-1 cells as described by Hogaboom *et al.* [27]. A partially purified enzyme (1 μmol O_2 consumed/min/mg protein) was obtained from gel filtration (TSK-4000 column, Beckman CA) and used directly. The 5-LO activity was the only fatty acid oxygenase activity in this preparation as evidenced by the synthesis of only 5-hydroperoxyeicosatetranoic acid (HPETE) and 5,6- and 5,12-dihydroxyeicosatetranoic acid (DHETE) isomers following the addition of arachidonic acid.

The assay for assessing inhibition of the 5-LO activity was a continuous assay that monitored the consumption of O_2. The stoichiometry for O_2 consumed and 5-HPETE/DHETE products was one at all times during the reaction. 5-LO (50 μg protein) was preincubated with the inhibitor or its vehicle in 25 mM bis-Tris buffer (pH 7.0) that contained 200 μM ATP, 5 μM sonicated dioloeyl phosphatidylcholine (Avanti Lipids, AL), 150 mM NaCl and 5% ethylene glycol for 2 min at 20° (total vol 2.98 mL). Arachidonic acid (10 μM) (Nuchek Prep, Eylsian, MN) and $CaCl_2$ (200 μM) were added to initiate the reaction (final vol. 3 mL). The decrease in O_2 concentration was followed with time using a Clark-type electrode with the Yellow Springs type $53O_2$ monitor (Yellow Springs, OH) and an offset amplifier (Sigma Electronic, King of Prussia, PA). The optimum velocity was calculated from the progress curves. Inhibitors were dissolved in ethanol with the final concentration of ethanol being 1% in the assay.

PGH Synthase activity. PGH synthase was purified from sheep seminal vesicles [28]. PGH synthase activity (15 μmol O_2 consumed/min/mg protein) was determined as described by Marshall *et al.* [29]. The assay buffer was 0.1 M potassium phosphate (pH 7.2), and the reaction was performed at 30°. Other additions included either 10 μM arachidonic acid or 50 μM arachidonic acid with the following combinations: 1 mM phenol and/or 1 mM glutathione with or without 1 unit of glutathione peroxidase. PGH synthase (20 nM) was added to start the reaction, and the decrease in O_2 concentration followed as described above for 5-LO. The optimum velocity was calculated from the progress curves. Inhibitors were dissolved in ethanol with the final concentration of ethanol being 1% in the assay.

12-Lipoxygenase activity (12-LO). 12-LO was obtained from the 200,000 *g* supernatant of human platelets. Platelet-rich plasma was prepared from whole blood by centrifuging for 40 min at 100 *g* using acidified citrate dextrose (1:6) as the anticoagulant. Plasma was acidified to pH 6.5 with 0.15 M citric acid and centrifuged at 900 *g* for 10 min. Platelets

were collected and washed twice with Tyrode's-HEPES buffer containing 2 mM EDTA and 5% ethylene glycol. The platelets (10 mL) were then disrupted by nitrogen cavitation using a Parr bomb at 750 psi for 10 min. The 200,000 *g* supernatant was collected and stored at −70° until assayed. No other fatty acid oxygenase activity was observed in this preparation as evidenced by the synthesis of only 12-HPETE and 12-HETE.

12-LO (20 μg protein) was added to 25 mM bis-Tris buffer (pH 7.0) that contained 2 mM $MgCl_2$ and 150 mM NaCl. Inhibitors or vehicle (1% ethanol final concentration) were preincubated for 1 min at 20° (total vol. 0.49 mL). Arachidonic acid (15 μM final concentration) was used to start the reaction (final vol. 0.5 mL). After 4 min (when the control reaction reached 50% of the total synthetic extent), the reaction was stopped with the addition of the internal standard 15-HETE (0.5 nmol) in 1 mL of cold ethyl acetate. The organic fraction was collected, dried under vacuum, and then resuspended with 50% methanol (0.4 mL). The amounts of 12-HETE and 12-HPETE were determined using reverse-phase HPLC techniques and by monitoring the effluent at 235 nm (see below). The sample was eluted with 50% acetonitrile buffered with 30 mM ammonium acetate (pH 5.8) with retention times of 8.7, 9.9 and 12 min for 15-HETE, 12-HETE and 12-HPETE, respectively.

Eicosanoid production by human monocytes and neutrophils. Human monocytes were isolated from peripheral blood using sedimentation on Ficoll followed by sedimentation on Percoll [30]. The monocyte fraction obtained from this technique was composed of >90% monocytes with the remainder being neutrophils, platelets and lymphocytes. Monocytes (10^7/mL) were placed into polypropylene tubes and used as a suspended culture. The assay buffer consisted of RPMI 1640 buffer, 1% human AB serum, 2 mM glutamine, 25 mM HEPES and 1 mM $CaCl_2$. The cells were preincubated for 5 min at 37° with constant agitation with the inhibitors. A23187 (2 μM) was added to stimulate eicosanoid production (final vol. 2 mL). After 5 min of incubation, PGB_2 (1 nmol) was added as an internal standard. The incubation buffer was collected by centrifugation (600 *g* for 15 min), and an equal volume of acetonitrile was added. Samples were stored at −70° until assayed. PGE_2 and LTC_4 were determined using radioimmunoassay (RIA) kits purchased from New England Nuclear (Boston, MA). The cross-reactivity of the LTC_4 RIA is 11.6% with LTD_4, 3.3% with LTE_4 and 0.03% with LTB_4. LTB_4, HHT and 12-HETE were analyzed using the extraction and chromatography techniques described below.

Neutrophils were isolated from freshly drawn heparinized venous human blood. Whole blood (25 mL) was layered over Ficoll (15 mL), and the mixture was centrifuged at 400 *g* for 30 min at room temperature. The red cell layer was collected and diluted with 35 mL of phosphate-buffered saline without Ca^{2+} and Mg^{2+} and layered over 6% Dextran (11 mL). The suspension was allowed to settle at room temperature for 45 min. The upper layer was collected, washed once, and then the remaining red cells were lysed by hypotonic shock. Neutrophils were washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+}. Neutrophils (10^7) were pretreated with SK&F 105561 or its vehicle [dimethyl sulfoxide (DMSO)] for 5 min at room temperature in 1 mL of phosphate-buffered saline containing Ca^{2+} (1 mM) and Mg^{2+} (1 mM). A23187 (10 μM) was added, and the mixture incubated for 5 min. PGB_2 (1 nmol) was added, which was followed by an equal volume of chilled acetonitrile to deproteinate the samples. Samples were centrifuged at 1000 *g* for 15 min at 5° and supernatants were extracted, chromatographed and analyzed for eicosanoid metabolites as described below.

Inhibition of eicosanoid production in rat whole blood. SK&F 105809 (or its carrier, 0.5% tragacanth) was administered orally at the doses described in the results. Cardiac blood was collected into 0.01% heparinized containers at various times after dosing. The total peripheral blood leukocyte count and the percent of neutrophils were determined.

Heparinized blood (3 mL) was placed into 15-mL polypropylene tubes and preincubated for 5 min at 37°. The stimulant, either zymosan (1 mg/mL) for LTB_4 production or A23187 (60 μM) for thromboxane B_2 (TxB_2) production, was added to initiate eicosanoid generation. Unstimulated aliquots of blood served as controls for background levels of eicosanoid production. Samples were incubated for 10 min at 37° with constant agitation. PGB_2 (1 nmol) was added followed immediately by ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA 10 mM) to stop the reaction. Blood samples were centrifuged at 400 *g* for 15 min. Plasma fraction was recovered, and 1 vol. of chilled acetonitrile was added. The suspension was centrifuged at 500 *g* for 10 min at 5°. Eicosanoid products in the supernatants were extracted and quantified as described below. LTB_4 production was corrected for neutrophil quantity.

Extraction and quantitation of eicosanoid metabolites. Samples for eicosanoid analysis were first extracted using solid phase extraction techniques and then analyzed using reverse-phase HPLC techniques. Samples were diluted with 1% formic acid and 1% triethylamine to reduce the acetonitrile to 20% and then loaded onto a preconditioned C18 SPE cartridge (J. T. Baker Chemical Co., Phillipsburg, NJ) at a flow rate of 1–2 mL/min. The cartridge was preconditioned according to the manufacturer's recommendations. The adsorbed samples were washed with 3 mL of aqueous 1% formic acid and 1% triethylamine, air dried, and then washed with 3 mL of petroleum ether. After air drying the cartridges, the eicosanoids were eluted with 3 mL of 70% acetonitrile containing 1% triethylamine. The solvent was removed under vacuum. Samples were resuspended in 300 μL of 30% acetonitrile buffered with 30 mM ammonium acetate (pH 5.8). The extraction recovery of eicosanoids was >90%.

The sample (200 μL) was loaded onto a reverse-phase column (RCM NOVA-PAK C_{18} 100 × 8 mm column, Waters Associates, Milford, MA). The starting mobile phase was 90% A [A = 10% acetonitrile buffered with 30 mM ammonium acetate

(pH 5.8)] and 10% B [B = 90% acetonitrile buffered with 30 mM ammonium acetate (pH 5.8)]. The flow rate for the separation was 2.5 mL/min. After 5 min, the %B was increased to 27% in a step fashion. Over the next 10 min, the %B was increased in a concave hyperbolic function (curve 9) to 40% and then increased in a linear manner to 60% over the next 10 min. Under these developing conditions, the retention times (min) for the eicosanoids were: 20-HO-LTB_4, 6; PGB_2, 10.5; transLTB_4, 12.5; LTB_4, 13.5; HHT, 17.2; 12-HETE, 20.4; and 5-HETE, 21. The HPLC system consisted of Waters 510 pumps, an 840 controller, a WISP injector and a 990 photodiode array detector.

The column effluent was monitored from 225 to 310 nm using a UV photodiode array detector. The eicosanoids in the sample were verified by their retention times and their UV absorbance spectra. The peaks were quantified by measuring their area of absorbance at their maximal wavelength of absorbance. This area was corrected to a mass unit using a standard curve generated from external standards. The calculated mass was corrected for the recovery of the internal standard. For TxB_2 measurement, the corresponding fraction (6.5 min) was collected and assayed by RIA (New England Nuclear).

Phospholipid turnover in human monocytes. Human monocytes were prepared as described above. The cells (5×10^6/mL) were labeled overnight with 10 μM [^{14}C]arachidonic acid (0.5 μCi/mL) (New England Nuclear) in polypropylene tubes at 37°. The incubation buffer was the same as described above for eicosanoid production from monocytes. At the end of the prelabeling period, the cells were harvested and washed twice with 1% fatty acid-free bovine serum albumin (BSA) (Sigma) in phosphate-buffered saline. Monocytes were resuspended to 15×10^6/mL in RPMI 1640 buffer containing 1% fatty acid-free BSA, 2 mM glutamine, 25 mM HEPES and 1 mM $CaCl_2$. SK&F 105561 or vehicle was preincubated for 10 min at 37° with constant agitation. A23187 (2 μM) was added (final incubation volume 1 mL), and the reaction stopped after 5 min by centrifugation at 5°. The buffer was removed and counted. Sedimented cells were extracted using the Bligh-Dyer technique [31]. The lipid fraction was chromatographed as described by Korte and Casey [32]. The developed chromatograms were scanned for radioactivity using a TLC radiodetector (Bioscan, Washington, DC).

Phospholipase activity. Porcine pancreatic phospholipase A_2 (PLA_2) was purchased from Sigma. Crude guinea pig lung phospholipase A_2 (PLA_2) activity was measured in the 100,000 *g* supernatant fraction of guinea pig lung as described by Bennett *et al.* [33]. Phosphatidylinositol-specific phospholipase C was partially purified from the U937 cells essentially as described for guinea pig tissue [34]. Peak II from the DEAE-Sepharose column was dialyzed overnight and purified further by heparin Sepharose chromatography. The enzyme from the heparin sepharose column utilized for the present study was enriched 260-fold. The inhibitors (dissolved in DMSO) were preincubated at a final concentration of 300 μM with the phospholipases for 10 min at 25°. The enzymes activities were then determined as described by Bennett *et al.* [33].

Antagonism of LTB_4 and LTD_4 binding to membranes. Antagonism of LTB_4 receptor binding was assessed using preparations of membranes from U937 cells as previously described by Winkler *et al.* [35]. Briefly, [^{3}H]LTB_4 (1 nM) (New England Nuclear) was incubated with the membrane preparation (50 μg protein) in a reaction buffer consisting of Tris–HCl (50 mM), $CaCl_2$ (10 mM) and $MgCl_2$ (10 mM) (pH 7.4) with and without the competing ligands for 30 min at 25°. The reaction was stopped by the addition of 20 vol. of ice-cold 10 mM Tris–HCl (pH 7.4). The bound and unbound [^{3}H]LTB_4 were separated by filtration on Whatman GF/C filters. The filter paper was counted to determine the extent of [^{3}H]LTB_4 binding to the membrane. Non-specific binding was the binding in the presence of 1 μM unlabeled LTB_4 and was <10% of the total binding.

Antagonism of LTD_4 receptor binding was assessed using membrane preparations from guinea pig lung as previously described by Mong *et al.* [36]. The incubation conditions were similar to those used for LTB_4 except that 0.25 nM [^{3}H]LTD_4 (New England Nuclear) was used as the radioligand. Non-specific binding was the binding in the presence of 1 μM unlabeled LTD_4 and was <15% of the total binding.

For both studies, the antagonists were dissolved in ethanol (1% final concentration) and were tested in a range from 10^{-7} to 10^{-4} M.

Evaluation of the effect of SK&F 105809 *and SK&F* 105561 *on human monocyte production of IL-1.* Human peripheral blood monocytes were isolated and purified from either fresh blood of volunteer donors or from Blood Bank buffy coat preparations (American Red Cross) according to an established protocol [37]. Purified monocytes were then seeded in 24-well plates at a concentration of 2 million cells/ml/well. The cells were allowed to adhere for 2 hr, nonadherent cells were removed by gentle washing, and test compounds were added to the cells for 1 hr before the addition of lipopolysaccharide (LPS) (10 ng/mL). The cultures were then incubated at 37° for an additional 18 hr, at which time culture supernatants were removed and assayed for IL-1 activity. IL-1 activity was measured by its ability to stimulate EL-4 cells to produce IL-2 as described previously [38]. A unit of activity is defined as the reciprocal of the dilution at which half-maximal response is achieved. To convert units into protein concentration, 500 units of IL-1 is equivalent to 1 ng of protein. All determinations of cytokine activity were made in comparison with a standard preparation maintained in our laboratory. Multiple experiments were performed and, where indicated, the results of a representative experiment are shown.

Quantitation of SK&F 105809 *and metabolites in rat blood samples.* Plasma concentrations of SK&F 105809 and the sulfide and sulfoxide metabolites, SK&F 105561 and SK&F 105942, were quantitated in rat blood after oral administration of SK&F 105809 (25–75 mg/kg). After a 30-min treatment period, cardiac blood was collected and a portion of

the blood was used for assessing eicosanoid production (see above). A 1-mL aliquot was used to determine the plasma levels of drug and metabolites. Plasma was prepared by centrifuging blood at 400 *g* for 30 min. An internal standard (SK&F 104493) was added to the plasma (100 μL), and the sample loaded onto an SPE C18 extraction cartridge (J. T. Baker Chemical Co.) that was preconditioned with methanol and water. The column was washed with 1 column volume of ammonium acetate (50 mM). SK&F 105809 and its metabolites were eluted from the column with methanol (2 × 500 μL). Methanol was evaporated under nitrogen, and the sample resuspended in HPLC mobile phase (100 μL). Aliquots (50 μL) of the reconstituted samples were quantitated on an HPLC system consisting of a model 6000A solvent delivery system, a model 590 solvent delivery system, a model 710B autoinjector, a Kratos Spectroflow 783 detector set at 270 nm, and a model RCM 8X10 radial compression module that contained a 5 μm NOVO-PAK C_{18} (5 × 100 mm) cartridge (Waters Associates). SK&F 105809 and its metabolites were separated by gradient elution with 10 mM ammonium acetate (solvent A) and 75% acetonitrile in 10 mM ammonium acetate (solvent B). Following injection, the percentage of solvent B was increased in a linear fashion over 10 min from 25 to 65%, and then held at 65% for an additional minute before returning to initial conditions. Flow rate was maintained at 2 mL/min throughout the analysis. Plasma concentrations of SK&F 105809 and metabolites were determined from peak area ratios (compared with internal standard) that were compared with standard curves that were made up in plasma and extracted.

For both SK&F 105809 and SK&F 105942, the assay had a limit of quantitation of 0.2 μg/mL and was linear up to 40 μg/mL. For SK&F 105561, the assay had a limit of quantitation of 0.05 μg/mL and was linear to 10 μg/mL. The coefficients of variation for analysis of SK&F 105809 at nominal concentrations (0.2, 2.0 and 20 μg/mL) in rat plasma were 12, 6, and 3%, respectively. The C-ratio (the ratio of the observed to the theoretical concentration) was determined to be in the range of 95 to 108%. The coefficients of variation for analysis of SK&F 105942 at nominal concentrations (0.2, 2.0 and 20 μg/mL) in rat plasma were 19, 5 and 3%, respectively. The C-ratio was determined to be in the range of 97 to 128%. The coefficients of variation for analysis of SK&F 105561 at nominal concentrations (0.05, 0.5 and 5 μg/mL) in rat plasma were 10, 3 and 3%, respectively. The C-ratio was determined to be in the range of 82 to 99%. SK&F 105809 and metabolites are stable in rat plasma for periods of up to 1 month when stored frozen at −20°.

RESULTS

Inhibition of 5-LO activity. The inhibition of 5-LO was studied using a partially-purified preparation because kinetic parameters (K_m and specific activity) were independent of protein levels, and the preparation was stable for several weeks. As observed with other preparations of 5-LO [39, 40], the progress curve for the enzyme was sigmoidal in shape having a distinct lag phase, an apparent steady-state velocity (optimal velocity) phase and a terminal phase due to enzyme inactivation. Therefore, an assay that continuously monitors the activity was used to determine the optimal velocity of the reaction. SK&F 105809 or the sulfoxide metabolite SK&F 105942 had no effect on this 5-LO activity (Table 1). However, the sulfide metabolite SK&F 105561 inhibited 5-LO with an IC_{50} of 3 μM (Table 1). SK&F 105561 was comparable in potency to NDGA, AA-861 and A63162, and was more potent than ETYA and phenidone. To decrease the lag time to optimal velocity, the amount of 5-LO was increased from 50 to 250 μg of protein which gave the corresponding 5-fold increase in activity. Phenidone, AA-861 and NDGA were less potent as the 5-LO activity increased, whereas the potency of SK&F 105561 was unchanged (Table 1). The potency of SK&F 105561 was also assessed in the absence of phosphatidylcholine liposomes since this cofactor may concentrate hydrophobic agents such as the substrate and the inhibitor. The enzyme concentration was increased 5-fold to compensate for the loss of the phospholipid stimulation of 5-LO. SK&F 105561 and phenidone inhibited with IC_{50} values of 5 and 20 μM, respectively (data not shown). It should be noted that SK&F 105561 or any other test compound did not lead to a change in oxygen levels and did not cause 5-LO to consume oxygen in the absence of arachidonic acid.

The mechanism of inhibition by SK&F 105561 was examined in greater detail. First, we found that the inhibition by SK&F 105561 was reversible. Pretreating the enzyme with 50 μM SK&F 105561 and then diluting 10-fold gave the same inhibition as pretreating with a final concentration of 5 μM (data not shown). Second, inhibition by SK&F 105561 did not increase with longer preincubation

Table 1. IC_{50} Determinations on 5-lipoxygenase and PGH synthase activities

Compound	IC_{50} [μM]	
	5-LO	PGH Synthase
SK&F 105561	3 (3)	100
SK&F 105809	NA	NA
SK&F 105942	NA	NA
AA-861	1 (6)	NT
A63162	3 (5)	NT
ETYA	20	NT
Naproxen	NA	80
NDGA	1 (5)	NT
Phenidone	15 (60)	Stimulates

NA represents not active at a concentration of 100 μM, and NT represents not tested. The concentration of the substrate (arachidonic acid) was 10 μM for both enzyme activities. The compound-mediated inhibition is described as the concentration of compound causing a 50% inhibition of the optimum velocity for the vehicle-treated sample. The values in parentheses represent the IC_{50} determined with a 5-fold greater 5-LO activity. Results are the means of three determinations. The standard deviations were less than 15% of the mean.

Table 2. Time-dependent effects of SK&F 105561 on 5-lipoxygenase inhibition

Preincubation time (min)	Velocity (nmol/min)		Inhibition (%)
	Control	SK&F 105561	
3	92	22	76
6	86	26	70
12	80	25	69
24	70	24	66

The partially purified 5-LO (100 μg) was preincubated in the presence of SK&F 105561 (10 μM) or vehicle in the reaction cuvette. Arachidonic acid (10 μM) and Ca^{2+} (200 μM) were added to start the reaction. The velocity of the reaction was measured. Time-dependent inhibition was measured with reference to the vehicle-treated control preincubated for the same time period.

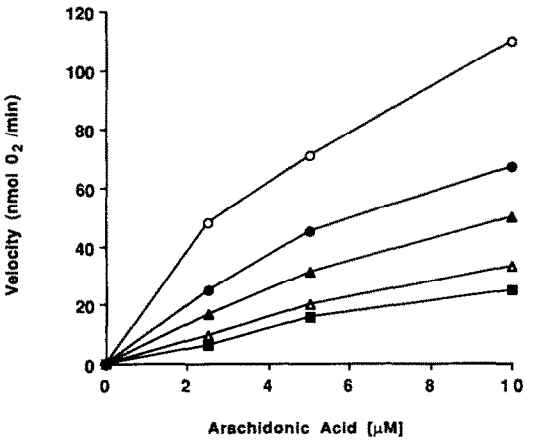


Fig. 2. Inhibition of 5-LO by SK&F 105561. The effect of arachidonic acid on the inhibition by SK&F 105561 (0–15 μM) was assessed using the partially purified 5-LO activity as described in Materials and Methods. The concentrations of SK&F 105561 were 0 μM (○), 2.5 μM (●), 5 μM (▲), 10 μM (△) and 15 μM (■). Each point is the average of two determinations.

times (Table 2). SK&F 105561 (10 μM) inhibited approximately 70% of the activity regardless of the preincubation time. Third, SK&F 105561 appeared to compete with the substrate (Fig. 2). The addition of arachidonic acid (2.5 μM) resulted in a velocity of 50 nmol O_2/min. In the presence of SK&F 105561, the concentration of arachidonic acid had to be increased to achieve a similar velocity (Fig. 2). The effect of SK&F 105561 on the maximal velocity could not be determined since concentrations of arachidonic acid greater than 10 μM resulted in an apparent substrate inhibition. However, the extent of the reaction was decreased with concentrations of SK&F 105561 greater than 5 μM (data not shown).

Inhibition of PGH synthase activity. PGH synthase is a single protein consisting of two activities: a cyclooxygenase activity, which uses arachidonic acid (K_m of 2 μM) to produce PGG_2, and a peroxidase activity, which reduces PGG_2 to PGH_2 [28]. Initial studies were performed under conditions where only the cyclooxygenase activity was expressed in order to determine the mechanism of inhibition of selected compounds. Naproxen inhibited the cyclooxygenase activity with an IC_{50} of 80 μM (Table 1). Additional studies indicated that naproxen was a competitive inhibitor with a K_i of 5 μM (data not shown). SK&F 105809 and SK&F 105942 at concentrations up to 100 μM did not affect the cyclooxygenase activity. SK&F 105561, however, had a biphasic effect on the cyclooxygenase activity. This compound stimulated the cyclooxygenase activity with 10 μM SK&F 105561, increasing activity 25% (data not shown). As the concentration of SK&F 105561 was increased, inhibition was observed with an IC_{50} of 100 μM (Table 1). Phenidone stimulated the cyclooxygenase activity in a concentration-related manner with 100 μM phenidone causing a 2-fold increase in cyclooxygenase activity (data not shown).

The effect of SK&F 105561 on PGH synthase activity was further explored in the presence of peroxidase activity. Arachidonic acid (50 μM) was used as the cyclooxygenase substrate to generate PGG_2, and phenol (1 mM) was used as a cosubstrate for the peroxidase activity to give the product PGH_2. Since the addition of phenol increased the cyclooxygenase activity approximately 8-fold, less PGH synthase was needed to follow the enzyme activity. In the absence of phenol, SK&F 105561 had a biphasic effect, stimulating the cyclooxygenase activity at low concentrations of compound and then inhibiting the activity at concentrations > 30 μM (Fig. 3). In the presence of phenol, SK&F 105561 inhibited in a concentration-related manner with an IC_{50} of 6 μM (Fig. 3). To further investigate the effect of increasing the peroxidase activity on the inhibition by SK&F 105561, glutathione peroxidase activity (1 unit) was included in the incubation. The addition of glutathione peroxidase resulted in an additional increase in the potency of SK&F 105561 with the IC_{50} being 3 μM (Fig. 3). The addition of glutathione with phenol had no additional effect over phenol alone on the potency of SK&F 105561 (Fig. 3).

Inhibition of 12-LO activity. The inhibition by SK&F 105561 on a third fatty acid oxygenase was determined using 12-LO activity from the 200,000 *g* supernatant of human platelets. This activity demonstrated a requirement for arachidonic acid (K_m 5 μM) and was stimulated 2-fold by Mg^{2+} (2 μM) but not by Ca^{2+}. SK&F 105561 had weak activity inhibiting 45% of the 12-LO activity at 200 μM (Fig. 4). ETYA demonstrated potent inhibition of this enzyme (IC_{50} 1 μM) (Fig. 4). Phenidone and SK&F 105809 were inactive at concentrations up to 200 μM (data not shown).

Inhibition of eicosanoid production from intact cells. The focus for the intact studies was to assess the relative inhibition of 5-LO, PGH synthase and 12-LO activities in the same setting. For these studies human monocytes and neutrophils were separately prepared from human blood.

The human monocyte preparation produced a variety of lipoxygenase and cyclooxygenase metabolites following A23187 stimulation. The major eicosanoid metabolites were LTB_4 for 5-LO,

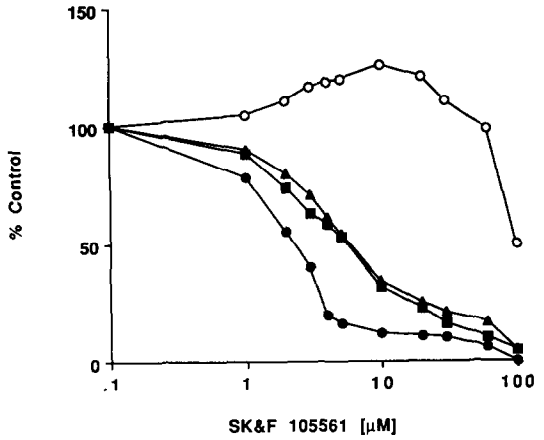


Fig. 3. Enhancement by glutathione peroxidase of the inhibition of PGH synthase by SK&F 105561. PGH synthase (20 nM) was incubated with various concentrations of SK&F 105561 and arachidonic acid (50 μM) (○). To assess the effect of peroxidase activity on the inhibition, PGH synthase (3.2 nM) was incubated with SK&F 105561, arachidonic acid (50 μM) and the following additions: phenol (1 mM) (■), phenol (1 mM)/glutathione (1 mM) (▲), or phenol (1 mM)/glutathione (1 mM)/glutathione peroxidase activity (1 unit) (●). The control value for no addition was 81 nmol O_2/min, for 1 mM phenol addition was 47.5 nmol O_2/min, for phenol and glutathione was 59 nmol O_2/min, and for phenol + glutathione + glutathione peroxidase was 32 nmol O_2/min. These values were used to calculate the percent changes shown.

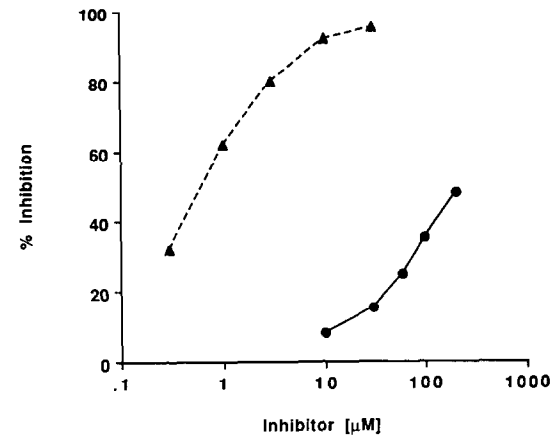


Fig. 4. Inhibition of 12-lipoxygenase. 12-LO activity was measured using the 200,000 *g* supernatant from purified platelets as described. The ETYA (▲) or SK&F 105561 (●) were preincubated with the enzyme for 1 min and then arachidonic acid (15 μM) was added to start the reaction. Product formation was measured after 4 min of incubation. The results are the means of three determinations, and the percent inhibition of vehicle-treated production at various concentrations of solution was determined. The controls for the three experiments used to calculate percent changes were 356, 760 and 2080 pmol of product.

Table 3. Inhibition of eicosanoid production from human monocytes

Compound	IC_{50} [μM]		
	LTB_4	PGE_2	12-HETE
SK&F 105561	2	0.1	140
SK&F 105809	NA	NA	NA
SK&F 105942	NA	NA	NA
ETYA	5	10	2
Phenidone	2	18	>100
Naproxen	37% at 40 μM	1	NA

NA refers to the compound showing no activity at a concentration of 100 μM. Human monocytes (10^7/mL) were pretreated with the compound for 5 min. Following an additional 10-min stimulation with A23187 (2 μM), the extracellular buffer was collected. Vehicle-treated monocytes produced 100 pmol LTB_4, 20 pmol PGE_2 and 20 pmol 12-HETE. Unstimulated cells produced no detectable eicosanoids. The compound-mediated inhibition is described as the concentration of the compound causing a 50% inhibition of the production for the vehicle-treated control. Results are the averages of two or more separate preparations of monocytes.

PGE_2 for PGH synthase, and 12-HETE for 12-LO. As with the enzyme preparations, SK&F 105809 and SK&F 105942 did not affect eicosanoid production in the intact monocytes (Table 3). SK&F 105561 inhibited the production of LTB_4 with an IC_{50} of 2 μM, being essentially equipotent with phenidone and ETYA (Table 3). Immunoreactive LTC_4 (15 pmol) was a minor product of A23187-stimulated monocytes and, likewise, was inhibited by SK&F 105561 and phenidone with similar IC_{50} values (data not shown). With regard to prostanoid production, SK&F 105561 potently inhibited PGE_2 production having an IC_{50} of 0.1 μM, which was 10-fold more potent than naproxen and 100-fold more potent than phenidone and ETYA (Table 3). Hydroxyheptadecatrienoic acid, a metabolite of the prostanoid pathway, was also inhibited with the same IC_{50} as PGE_2 produciton (data not shown). The production of 12-HETE was inhibited weakly by SK&F 105561, requiring 140 μM to achieve 50% inhibition (Table 3). ETYA was the most potent agent tested, inhibiting 12-HETE production with an IC_{50} of 2 μM. The potency of SK&F 105561 was not changed by increasing the cell number. Stimulating monocytes with 2 μM A23187, SK&F 105561 inhibited LTB_4 production from 0.5×10^6 monocytes or 20×10^6 monocytes with an IC_{50} of 2 μM (data not shown). The addition of arachidonic acid (30 μM), however, caused a 20-fold decrease in the potency of SK&F 105561 on LTB_4 production (data not shown). SK&F 105561 (50 μM) had no effect on cell viability as measured by trypan blue exclusion during 1 hr of incubation at 37° (data not shown).

The human neutrophil was examined to determine if SK&F 105561 inhibited both 5-HETE and LTB_4 production with the same potency. Unstimulated cells failed to produce any 5-LO metabolites. The addition of A23187 (10 μM) resulted in the production of both the 5-LO metabolite 5-HETE and the LTA_4

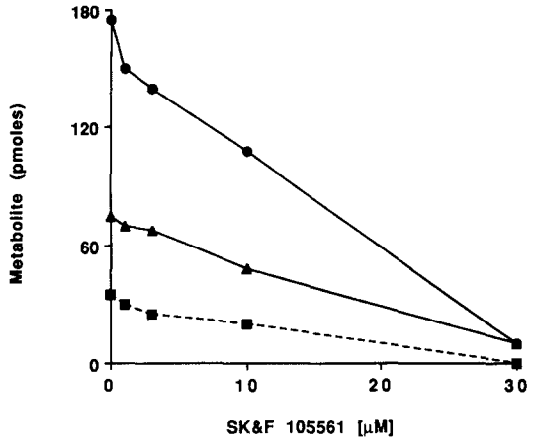


Fig. 5. Inhibition of LTB_4 production from human neutrophils. Human neutrophils (10^7/mL) were pretreated with SK&F 105561 and then stimulated with A23187 (10 μM) as described in Materials and Methods. LTB_4 (▲), 5-HETE (■) and total 5-LO products (LTB_4, transLTB$_4$, 20-hydroxyLTB$_4$ and 5-HETE) (●) were quantified as described in Materials and Methods.

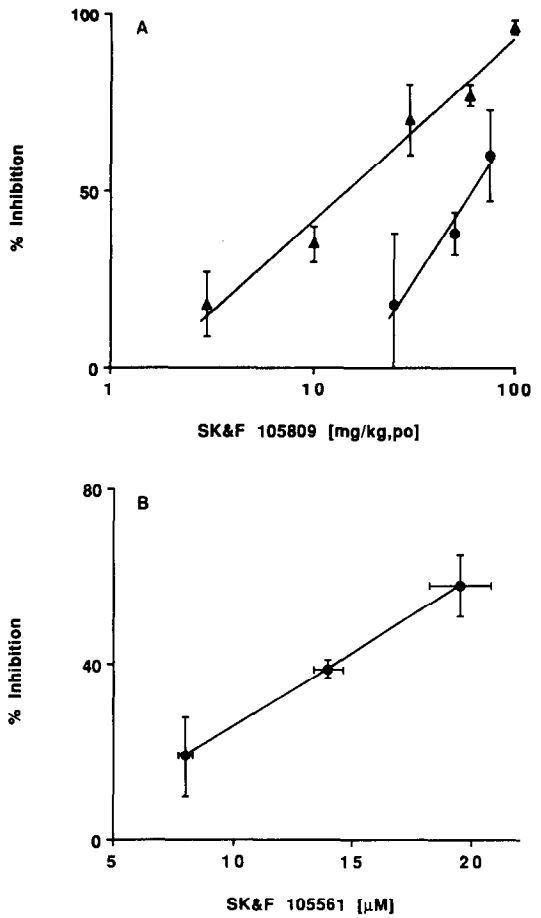


Fig. 6. (A) SK&F 105809-mediated inhibition of LTB_4 and TxB_2 production *ex vivo*. SK&F 105809 was administered orally at the indicated doses to rats. After a 1-hr dosing, blood was collected and stimulated with either zymosan to produce LTB_4 (●) or A23187 to produce TxB_2 (▲) as described in Materials and Methods. The percent inhibition was determined with respect to the production in blood that was obtained from vehicle-treated rats. Values are the means (± SD) for five rats per dose. The control value for TxB_2 was 627 ± 82 pmol measured as HHT and the control value for LTB_4 was 12.5 pmol/10^6 polymorphonuclear leukocytes (PMN). (B) Correlation of the plasma levels of SK&F 105561 with the inhibition of LTB_4 *ex vivo*. In a separate experiment, blood was obtained from rats that had been orally dosed with SK&F 105809 (25, 50 and 75 mg/kg) and analyzed for the presence of SK&F 105561. The resulting mean level of SK&F 105561 was plotted against the observed inhibition of LTB_4 production. The percent inhibition was determined with respect to the production in blood that was obtained from vehicle-treated rats. The resultant inhibition represents the mean (± SD) from five rats. The control value for the zymosan stimulated, vehicle-treated rats was 12.5 ± 3.3 pmol/10^6 PMN.

metabolites 20-hydroxyLTB$_4$, LTB_4 and 5,12-transLTB$_4$ with the sum of all metabolites being 175 ± 25 pmol/10^6 neutrophils. SK&F 105561 did not display any preferential inhibition of either the dioxygenase of LTA_4 synthase activities since the IC_{50} values on 5-HETE and LTA_4 metabolites were both 10 μM (Fig. 5).

Inhibition of eicosanoid production from rat blood ex vivo. The *ex vivo* production of eicosanoids was evaluated in whole blood that was collected from rats following the oral administration of SK&F 105809. The predominant metabolites observed were the 5-LO metabolite, LTB_4 (which is mainly synthesized by neutrophils) and the cyclooxygenase metabolites, TxB_2 and HHT (which are mainly synthesized by platelets). These metabolites of arachidonic acid can be produced simultaneously or separately depending on the stimulus. Studies were done using stimulus specific for each eicosanoid, zymosan for LTB_4 production and A23187 for TxB_2 and HHT. Zymosan was somewhat less effective as a stimulus for LTB_4 production than A23187 (8 vs 20 pmol of LTB_4/mL of blood). Zymosan did not stimulate the synthesis of TxB_2 or HHT much above background (<4 pmol/mL of blood), whereas A23187 provoked a 20-fold increase in TxB_2 and HHT production.

Rats were treated orally with SK&F 105809 for 1 hr. Blood was collected and stimulated with zymosan (1 mg/mL). SK&F 105809 inhibited LTB_4 production in a dose-dependent manner with an ED_{50} of 60 mg/kg (Fig. 6A). Plotting the blood levels of SK&F 105561 against the degree of inhibition of LTB_4 production demonstrated that a blood level of 15 μM SK&F 105561 correlated with a 50% inhibition of LTB_4 production (Fig. 6B).

Using A23187 as a stimulus, SK&F 105809 inhibited both TxB_2 and HHT production in a dose-dependant manner with an ED_{50} of 15 mg/kg (Fig. 6A). However, the ability of SK&F 105809 to inhibit LTB_4 production was reduced with the ED_{50} being greater than 100 mg/kg, p.o. (data not shown).

Phospholipase activity and phospholipid turnover. The inhibition of eicosanoid production in intact cells could also be attributed to an inhibition of

Table 4. Effect of SK&F 105561 on phospholipid turnover in human monocytes

Treatment	Radioactivity (cpm)			
	PC	PI/PS	PE	Total cellular
Unstimulated	7400	6000	800	15,000
Stimulated	5800 ± 300	5630 ± 900	850 ± 80	14,100 ± 400
SK&F 105561				
1 μM	5700 ± 300	5500 ± 200	700 ± 160	14,000 ± 600
10 μM	5800 ± 350	5700 ± 200	730 ± 40	14,500 ± 1000
30 μM	6200 ± 600	5800 ± 400	640 ± 70	14,100 ± 1400

Monocytes were prepared and prelabeled with 10 μM [^{14}C]arachidonic acid (sp. act. 55 Ci/mol) overnight. The cells were stimulated with A23187 (2 μM) for 10 min. The buffer and cells were separated and analyzed. Results are the means ± SD for four determinations.

phospholipase activity. Indeed, one explanation for the reversal of inhibition by added arachidonic acid could be due to circumventing the phospholipase inhibition. The possible inhibitory actions of SK&F 105809 and SK&F 105561 were examined using phospholipase A_2 activity from guinea pig lung and the phosphatidylinositol-specific phospholipase C from U937 cells. At a test dose of 300 μM, neither SK&F 105561 nor SK&F 105809 had any effect on either of the phospholipase activities (data not shown).

The mechanism for arachidonic acid mobilization used by the human monocyte may be different than via the phospholipase activities studied above. Therefore, the arachidonic acid turnover was examined in intact monocytes. The monocytes were prelabelled with [^{14}C]arachidonic acid overnight in order to achieve apparent steady-state labeling of the lipid pools. SK&F 105561 from 1 to 30 μM had no effect on the A23187-stimulated turnover of arachidonic acid in the major phospholipid pools (Table 4). The stimulated release of radioactivity (3300 ± 1000 cpm) into the incubation buffer was not affected by SK&F 105561 treatment.

Antagonism of leukotriene binding. Several classes of 5-LO inhibitors have been shown to block leukotriene binding to their respective receptors [41, 42]. SK&F 105561 and SK&F 105809 did not antagonize the binding of either LTB_4 or LTD_4 to their respective membrane receptors.

Inhibition of IL-1 production. Since SK&F 105561 dramatically reduced the production of eicosanoids by stimulated human monocytes, it was of interest to determine whether other inflammatory mediators were being affected as well. As seen in Table 5, SK&F 105561, but not SK&F 105809, markedly inhibited the produciton of IL-1 from LPS-stimulated human monocytes. Maximal inhibition observed was seen at a 10 μM concentration of SK&F 105561 and the inhibition was concentration-related with an IC_{50} of 2 μM.

Table 5. Effect of SK&F 105561 on interleukin-1 production in human monocytes

Treatment	IL-1 β (pg/mL)	Percent inhibition
SK&F 105561		
10 μM	1004 ± 45	78.7
5	1433 ± 121	69.6
1	2697 ± 211	42.9
0.5	3516 ± 232	25.5
0.1	4745 ± 332	0.0
0.0	4722 ± 332	
SK&F 105809		
10 μM	3908 ± 180	15.7
5	4446 ± 220	4.1
1	4703 ± 237	0.0
0.5	4516 ± 342	2.6
0.1	4719 ± 332	0.0
0.0	4638 ± 453	

Human monocytes (2×10^6 cells/mL/well) were prepared as described and stimulated with LPS (10 ng/mL). Culture supernatants were harvested 19 hr post-culture. Compounds were added 1 hr before addition of LPS. IL-1 activity was assayed as described. Data are means ± SD.

DISCUSSION

SK&F 105809 and the sulfoxide metabolite SK&F 105942 have no demonstrable effect on *in vitro* eicosanoid production. However, the sulfide metabolite SK&F 105561 did inhibit eicosanoid synthesis which can be attributed to its ability to inhibit the enzymatic activities of both 5-LO and PGH synthase.

The inhibition of 5-LO activity was assessed using the partially purified enzyme because the K_m for substrate was independent of protein concentration. The apparent K_m for arachidonic acid was 10 μM with an apparent substrate inhibition being observed as the concentration increased. SK&F 105561 inhibited this activity with an IC_{50} of 3 μM. This inhibition was reversible and was not dependent on the length of pretreatment. Moreover, SK&F 105561 appeared to protect the enzyme from inactivation during the preincubation peroid. The control activity decreased from 92 to 70 nmol/min over the 24-min preincubation. In the presence of SK&F 105561 (10 μM), the 5-LO activity appeared to remain constant. This property was not shared by other

inhibitors such as phenidone, dithiothreitol or NDGA (data not shown). The mechanism for the protection by SK&F 105561 is unknown, It may well result from neutralizing a toxic factor in the incubation buffer or from a direct interaction with 5-LO and thus preventing a modification of the enzyme that results in inactivation.

SK&F 105561 appeared to be affecting both the maximal observed velocity of the enzyme and the K_m for arachidonic acid although the exact mechanism of action could not be determined. Part of the difficulty in establishing the mechanism of inhibition is due to the narrow range of substrate concentrations (2.5 to 10 μM) that can be used. A second and more fundamental limitation is that a steady-state condition may not be achieved despite the appearance of a linear reaction progress curve. Even though the maximal slope of the reaction was used to calculate the optimal velocity, the reaction profile for 5-LO is complex and limited in duration. The potency of inhibition by SK&F 105561 was inversely related to the arachidonic acid concentration which suggests a competitive component to the inhibition. However, the maximal observed velocity as well as the extent of the reaction was noticeably decreased with the higher concentrations of inhibitor. SK&F 105561 may have extended the activation phase of the reaction and/or slowed the catalytic phase. Since inactivation of the enzyme is undoubtedly occurring during this time, the result of the inhibition by SK&F 105561 would be the loss of catalytic activity and thus an apparent decrease in V_{max}. The addition of lipid hydroperoxides has been used to minimize the lag phase [40, 43]. However, lipid hydroperoxides also cause the enzyme to inactivate and terminate the enzyme reaction more quickly [44, 45]. We have avoided using exogenous hydroperoxides; instead, we have used concentrations of the enzyme that will autoactivate quickly and demonstrate linear increases in velocity with increasing enzyme concentrations. Despite these efforts, mechanistic conclusions regarding SK&F 105561 would require a more rigorous approach examining each phase of the reaction profile.

The inhibition of PGH synthase by SK&F 105561 depended on the presence of peroxidase activity. The addition of 1 unit of glutathione peroxidase improved the potency of SK&F 105561 from an IC_{50} of 100 μM to 3 μM. Similar results have been reported for a variety of non-steroidal antiinflammatory drugs (e.g. acetoamidophenol, meclofenamic acid, and phenylbutazone) [46, 47]. These agents appear to remove a radical intermediate that is essential for cyclooxygenase activity. The generation of this radical is dependent on the hydroperoxide concentration. Thus, these types of compounds become more effective inhibitors when the activating hydroperoxides for cyclooxygenase is decreased. As a consequence of this mechanism of inhibition, predictions regarding the effectiveness of SK&F 105561 for inhibiting PG production must consider both the peroxide-producing and peroxide-removing capability of the tissue. This observation provides a rationale for the variation in IC_{50} values between the various test systems.

The ability of SK&F 105561 to inhibit 5-LO and PGH synthase appeared not to be the result of its antioxidant/reduction potential. The reduction potential for SK&F 105561 was 0.7 V as compared to 0.3 V for phenidone. Both compounds inhibited 5-LO with similar potency. With respect to other pyrroloimidazone compounds, there was also no correlation between the reduction potential (which ranged from 0.3 to 1 V) and their potency on either 5-LO or PGH synthase (data not shown).

Eicosanoid production in the intact cell was assessed using both human monocytes and human neutrophils. They represent two cell-types having different levels of fatty acid oxygenase activities with the possibility of having different controls on the expression of these activities. Stimulation of the neutrophils resulted in the production of both 5-HETE and LTA_4 metabolites. SK&F 105561 inhibited the production of these metabolites with IC_{50} values of 10 μM. The human monocyte preparation has 5-LO, PGH synthase and 12-LO activities with the latter activity most likely due to contaminating platelets. SK&F 105561 inhibited the synthesis of PGE_2, LTB_4, and 12-HETE with IC_{50} values of 0.1, 2, and 140 μM, respectively. It should be noted that HHT and PGE_2 production was inhibited by SK&F 105561 with similar IC_{50} values as was the synthesis of LTB_4 and LTC_4. These results suggest that SK&F 105561 was inhibiting the first committed enzyme in each of the respective pathways as opposed to inhibiting the release of substrate of subsequent metabolic steps. The potency of inhibition was not diminished by increasing the cell concentration as has been observed with other 5-LO inhibitors [48].

For eicosanoid synthesis to occur, free arachidonic acid must be made available to the fatty acid oxygenases [49]. It is thought that this occurs through the action of phospholipase A_2 to release arachidonic acid from phospholipids. Antiinflammatory corticosteroids are thought to work by inhibiting phospholipase activity [1–4]. SK&F 105561 did not inhibit several preparations of isolated phospholipase activity, nor did it affect the A23187-stimulated turnover of arachidonic acid from prelabeled human monocytes. Furthermore, SK&F 105561 inhibited LTB_4 and PGE_2 production following the addition of arachidonic acid (30 μM) to A23187-stimulated monocytes, although the IC_{50} increased from 2 to 30 μM (unpublished observation). This decrease in potency following the addition of arachidonic acid is consistent with a competitive inhibition of the fatty acid oxygenases. The inhibition of eicosanoid metabolism from intact cells is most likely due to the direct inhibition of the fatty acid oxygenases by SK&F 105561.

The oral administration of SK&F 105809 resulted in a dose-related inhibition of both leukotriene and prostanoid products as measured in rat blood *ex vivo*. Although SK&F 105809 inhibited both the 5-LO and PGH synthase pathways using A23187 as the stimulus for human blood, there was the concern that the greater potency of SK&F 105809 on the PGH synthase pathway could be masking the inhibition of the 5-LO pathway via the shunting of arachidonic substrate. Following A23187 stimulation of whole blood from rat, there is 5- to 10-fold

more prostanoid metabolites produced than 5-LO metabolites. Inhibition of the PGH synthase activity could result in a greater amount of substrate being available for the 5-LO pathway to compete with SK&F 105561 [50]. Indeed, inhibition of the PGH synthase pathway has been shown to lead to enhanced leukotriene production [51–53]. Our results from the experiments with monocytes demonstrated that the potency of SK&F 105561 was reduced by increasing the substrate concentration. Therefore, specific stimulation of the 5-LO pathway was desirable. In the blood, the platelet has most of the PGH synthase and 12-LO activities, and the neutrophil has most of the 5-LO activity. The neutrophil can be specifically stimulated by zymosan through the direct binding to the β-glucan receptor on the neutrophil or indirectly through the activation of complement. Zymosan was used to selectively stimulate the 5-LO pathway, and A23187 was used to stimulate both the 5-LO and PGH synthase pathways. With this protocol, SK&F 105809 inhibited both eicosanoid pathways in a dose-related fashion. The inhibition of TxB_2 production following A23187-stimulation was observed with an ED_{50} of 15 mg/kg, p.o., of SK&F 105809. The inhibition of LTB_4 production following zymosan-stimulation *ex vivo* was observed with an ED_{50} of 60 mg/kg, p.o. This dose of SK&F 105809 resulted in a blood concentration of SK&F 105561 equal to 15 μM, a concentration which inhibited LTB_4 production by 50%.

An additional aspect of the activity of SK&F 105561 is its ability to inhibit the production of the inflammatory cytokine, IL-1. This activity is among the most potent effects of SK&F 105561. While the mechanism is not known, it is not associated with inhibition of fatty acid oxygenases, since neither the 5-LO inhibitor, phenidone, nor the CO inhibitor, naproxen, inhibits IL-1 production [54]. In addition, recent evidence indicates that SK&F 105561 inhibits TNF production as well. That cytokine inhibition is of relevance to the *in vivo* pharmacology of SK&F 105809 is indicated by the observation that TNF production was inhibited with an ED_{50} of 30 mg/kg, p.o. in LPS-treated animals.* Thus, this property likely contributes to the antiinflammatory activity of SK&F 105809. Work to determine the mechanism of cytokine inhibition is continuing.

These data demonstrate the dual inhibition of 5-LO and PGH synthase by SK&F 105561, the active metabolite of SK&F 105809. The ability of SK&F 105561 to inhibit both eicosanoid pathways and to inhibit the production of IL-1 and TNF suggests that this active metabolite of SK&F 105809 will possess antiinflammatory activity similar to that of antiinflammatory corticosteroids. The *in vivo* pharmacology profile of SK&F 105809 and its activity in animal models of inflammation are described in the companion paper [25].

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* Olivera D, Lee JC and Badger AM, Inhibition of serum tumor necrosis factor and protection from lethality in a murine model of endotoxin shock by SK&F 105809, a cytokine inhibitor (manuscript in preparation).

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