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CONTRASTING EFFECTS OF TWO ARACHIDONATE 5-LIPOXYGENASE INHIBITORS ON FORMYL-METHIONYL-LEUCYL-PHENYLALANINE (fMLP) AND COMPLEMENT FRAGMENT 5a INDUCED HUMAN NEUTROPHIL SUPEROXIDE GENERATION

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Abstract—SC-45662 and SC-41661A, selective arachidonate 5-lipoxygenase (5-LO) inhibitors, had markedly different effects on formyl-methionyl-leucyl-phenylalanine (fMLP) and complement fragment 5a (C5a) induced superoxide release from human neutrophils (PMNs). SC-45662 inhibited superoxide generation induced by fMLP and C5a with IC_{50} values of 12 and 5 μM, respectively. Furthermore, SC-45662 was capable of inhibiting fMLP and C5a induced superoxide release in PMNs primed with bacterial lipopolysaccharide, tumor necrosis factor-α and other priming agents. SC-41661A, a compound from the same chemical series as SC-45662, did not inhibit or induce superoxide generation, but instead primed PMNs for fMLP and C5a induced superoxide generation. The induced superoxide release was concentration dependently enhanced 2 to 4-fold at 5–50 μM. Superoxide release induced by phorbol myristate acetate or serum-activated zymosan was unaffected by either SC-45662 or SC-41661A. The regulation of superoxide generation by these compounds, both of which have the identical oxidation–reduction pharmacophore, was clearly independent of their effects on 5-LO activity. Furthermore, the mechanism by which SC-45662 and SC-41661A alter superoxide generation did not appear to depend on inhibition of xanthine oxidase, catalase or superoxide dismutase. These new compounds provide effective tools for further investigation of the relationship of these two biochemical oxidative systems.

Key words: superoxide; 5-lipoxygenase; SC-45662; SC-41661A; neutrophil; priming

Activated phagocytic cells generate lipoxygenase products and oxygen radicals in response to similar inflammatory stimuli [1–7]; however, the relationship between these systems has not yet been clarified. LTB_4‡, a product of the 5-LO pathway, is chemotactic for inflammatory cells, enhances neutrophil attachment to endothelial cells and induces neutrophils to degranulate and release proteolytic enzymes [1–5]. The peptidoleukotrienes—LTC_4, LTD_4 and LTE_4—are potent mediators of bronchoconstriction, mucus formation, and microvascular leakage associated with asthma [2]. The leukotrienes have also been shown to stimulate the synthesis of interleukin-1, a cytokine associated with numerous properties of the inflammatory process [8–10]. Superoxide and other reactive oxygen species derived from superoxide have been associated with joint inflammation and cartilage destruction in rheumatoid arthritis [11–13]. Oxygen radicals have also been implicated in the pathogenesis of septic shock, the adult respiratory distress syndrome, asthma and ischemia/reperfusion injury in a variety of organs [6, 7, 14–19]. The recent emphasis on the role of oxygen radicals in these and other pathological conditions has prompted the discovery of new antioxidant compounds to be used as possible therapeutic agents to treat these conditions and as biological tools to promote further investigation of the role of oxygen radicals [20].

SC-45662, ±[2*S**-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]1*R**-methylpropoxy]acetic acid, and SC-41661A, 3-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thiol]-*N*-methyl-*N*-[2-(2-pyridinyl)-ethyl]propanamide (Fig. 1) were synthesized as part of our program to discover selective, anti-oxidant-type 5-LO inhibitors. Both compounds inhibited arachidonate 5-LO from RBL-1 cells and the release of 5-LO products from human PMNs with little or no effect on CO, 12-LO, 15-LO or PLA_2. The compounds were tested further to determine if they could also affect superoxide generation in both primed and unprimed human PMNs. We, herein, present data to show that while both SC-45662 and SC-41661A selectively inhibited

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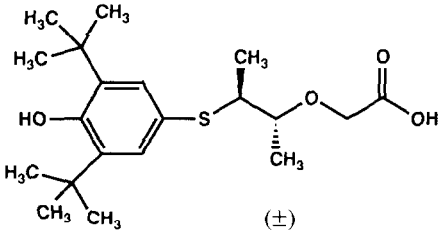
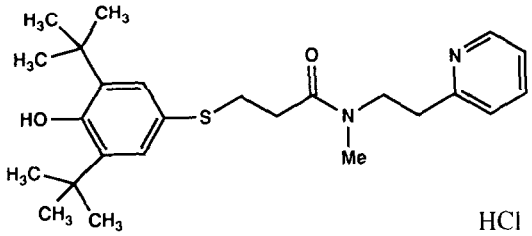
‡ Abbreviations: LT, leukotriene; LO, lipoxygenase; RBL-1, rat basophilic leukemia; PMNs, polymorphonuclear leukocytes, neutrophils; fMLP, formyl-methionyl-leucyl-phenylalanine; C5a, complement fragment 5a; AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; PMA, phorbol myristate acetate; CO, cyclooxygenase; and PLA_2, phospholipase A_2.

SC-45662



(±)

SC-41661A



HCl

Fig. 1. Structures of SC-45662 and SC-41661A. SC-45662, ±[2*S**-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio] 1*R**-methylpropoxy]acetic acid, and SC-41661A, 3-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-*N*-methyl-*N*-[2-(2-pyridinyl)ethyl]propanamide.

5-LO and contained the same redox pharmacophore, they had markedly opposite effects on superoxide generation.

MATERIALS AND METHODS

Compounds. SC-45662 and SC-41661A were synthesized at Searle Research and Development, Skokie, IL.

Cell-free rat basophilic leukemia cell 5-lipoxygenase assay. Cell-free 5-lipoxygenase activity isolated from RBL-1 cells was quantified by the method of Jakschik *et al.* [21]. RBL-1 cells were suspended in 10 mM HEPES, pH 7.4, containing 1 mM EDTA, 0.1% (w/v) gelatin and 17 μM indomethacin, homogenized and centrifuged at 100,000 *g* for 30 min. The supernatant, containing 2.25×10^7 cell equivalents/mL HEPES buffer supplemented with 4 mM $CaCl_2$, was preincubated with test compound for 8 min prior to the addition of 36 μM [^{14}C]AA (54.6 mCi/mmol, New England Nuclear, Wilmington, DE) for 5 min at 37°. The products were extracted twice with diethyl ether and isolated by TLC (hexane:2-propanol:acetic acid, 229:20:1). The [^{14}C]5-HETE spot was removed and quantified by liquid scintillation counting. Corrections were made based on the recovery of a [^{3}H]prostaglandin D_2 internal standard.

Intact RBL-1 cell 5-lipoxygenase assay. Five million intact RBL-1 cells/mL of Eagle's medium were preincubated at 37° with test compound for 5 min prior to the addition of the calcium ionophore A23187 (5 μg/mL, Calbiochem, La Jolla, CA). Thirty minutes later, the cells were removed by centrifugation, and the LTC_4 released into the medium was quantified by radioimmunoassay (New England Nuclear, Boston, MA).

Intact human neutrophil 5-lipoxygenase assay. Human neutrophils (>95% purity) were isolated from peripheral blood by dextran sedimentation, Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation, and hypotonic lysis [22]. Ten million PMNs/mL of Hanks' balanced salt solution containing $CaCl_2$ were preincubated with test compound for 5 min prior to the addition of the calcium ionophore A23187 (0.2 μg/mL) and 9 μM [^{14}C]AA to initiate the 5-min reaction at 37°. The products were extracted and eluted on a Beckman HPLC Ultrasphere ODS column (5 μm, 4.6 × 250 mm) isocratically for 20 min (methanol:water: acetic acid, 60:40:0.05, pH 5.4, adjusted with ammonium hydroxide) and then switched to 100% methanol over 60 min followed by a 20-min washout with 100% methanol at a flow rate of 1 mL/min. The eluate was monitored for hydroxy-fatty acids (235 nm), leukotrienes (280 nm) and radioactivity (in line Flo-One/Beta radioactivity detector).

Human platelet 12-lipoxygenase assay. Human platelets, isolated by the method of Yen and Morris [23], were suspended in 15 mM Tris/1 mM EDTA/isotonic saline buffer, pH 7.4, lysed by freeze–thawing three times and centrifuged at 40,000 *g* for 30 min. The supernatant fraction, containing 3×10^8 cell equivalents/mL of Tris buffer supplemented with 2 mM $CaCl_2$, was preincubated with test compound for 5 min at 37° prior to the addition of 36 μM [^{14}C]AA. After 3 min, the products were extracted and isolated by TLC (organic phase of ethyl acetate:isooctane:acetic acid:water, 110:50:20:100). The [^{14}C]12-HETE was quantified by liquid scintillation counting.

Soybean 15-lipoxygenase, cyclooxygenase and phospholipase A_2 assays. 15-LO, CO and PLA_2 activities were quantified by monitoring oxygen consumption as described by Wallach and Brown [24]. Briefly, soybean 15-LO (Sigma) and test compound were preincubated in 0.1 M 2-amino-2-methyl-1,3-propanediol–HCl buffer, pH 8.4 (Ammediol buffer), at 37° for 5 min. A 50 μM concentration of sodium AA (Nu Chek Prep, Elysian, MN) was added to start the reaction. For the CO assay, lyophilized ovine seminal vesicle microsomes (Pel-Freeze Biologicals, Rogers, AK) in 0.25 M Tris–HCl buffer, pH 8.2, containing 0.7 mM phenol were preincubated with test compound for 5 min at 37° prior to the addition of 50 μM sodium AA. For the PLA_2 assay, the fatty acids hydrolyzed by PLA_2 from the 2-position of the glycerol moiety of phosphatidylcholine were quantified by measuring the oxygenation of the fatty acids catalyzed by soybean 15-LO. Hog pancreatic PLA_2 (Sigma), soybean 15-LO and test compound were preincubated for 5 min at 37° in Ammediol buffer containing 0.13 mM $CaCl_2$. Phosphatidylcholine (Sigma) prepared in 0.5% deoxycholate was added to initiate the reaction.

Human neutrophil superoxide generation assay. Superoxide generation was quantified by the

superoxide dismutase inhibitable reduction of ferricytochrome *c* [25]. Freshly isolated human PMNs (2×10^6)/mL of Krebs–Ringer phosphate buffer, pH 7.2, were incubated with inhibitor and 80 μM ferricytochrome *c* (horse heart-III, Sigma) for 5 min at 37°. In some experiments, PMNs were also preincubated with priming agents which included 2×10^7U/mg TNF-α (human recombinant, Genzyme, Boston, MA) or 5 nM PMA (Sigma) for 10 min, or 50 ng/mL bacterial LPS *Escherichia coli* 0111; B4, Sigma) for 30 min. fMLP (0.1 μM, Sigma), 10 nM human recombinant C5a (Sigma), 40 nM PMA or serum-activated zymosan (0.75 mg/mL, Sigma) was added to induce superoxide generation. The change in absorption at 550 nm was recorded every 30 sec for 10 min. The final absorbance reading minus the initial reading was used to calculate nanomoles of superoxide generated based on a molar extinction coefficient of 2.1×10^4 cm^{-1} mol^{-1} [25]. PMNs from a single donor were used to generate a concentration–response curve since superoxide levels generated by different donors showed considerable variation. The data shown are representative of at least three experiments, using different blood donors.

[^{3}H]*fMLP binding to human neutrophils*. The method of Abita [26] was used to measure fMLP binding to intact human neutrophil receptors. Two million neutrophils/mL of Krebs–Ringer phosphate buffer, pH 7.2, containing 2 mg/mL dextrose were preincubated with unlabeled fMLP, SC-45662 or SC-41661A for 10 min at room temperature prior to the addition of 20 nM [^{3}H]fMLP (56.9 Ci/mmol, New England Nuclear). Thirty minutes later, the samples were chilled and centrifuged at 12,000 *g* for 30 sec. The radioactivity in the cell pellet was determined by liquid scintillation counting. Non-specific binding (1000 × unlabeled-fMLP relative to [^{3}H]fMLP) was subtracted from each sample.

Xanthine oxidase, superoxide dismutase and catalase assays. Xanthine oxidase activity was measured by quantifying ferricytochrome *c* reduction of superoxide generated by a cell-free xanthine/xanthine oxidase system [27]. Superoxide dismutase activity was measured by its capacity to inhibit ferricytochrome *c* reduction by superoxide generated in the xanthine/xanthine oxidase system described above. Compounds were added to a calibrated system in which superoxide dismutase inhibited ferrycytochrome *c* reduction by approximately 60–70%. Catalase activity was monitored by measuring oxygen liberation from hydrogen peroxide into oxygen-free buffer [28].

RESULTS

Inhibition of arachidonate 5-lipoxygenases by SC-45662 and SC-41661A. SC-45662 and SC-41661A inhibited the conversion of [^{14}C]AA to 5-HETE by the 100,000 *g* supernatant of RBL-1 cell homogenate. The acidic derivative, SC-45662, had a half-maximal inhibitory concentration (IC_{50}) of 3.5 μM, whereas the basic compound, SC-41661A, was 10-fold more active with an IC_{50} of 0.3 μM, as shown in Table 1.

The production of LTC_4 by calcium ionophore A23187-stimulated RBL-1 cells was used to assess the abilities of SC-45662 and SC-41661A to inhibit intact cell 5-LO (Table 1). Compounds were added to cell incubates 5 min prior to the addition of the ionophore. The IC_{50} values for SC-45662 and SC-41661A were 11.4 and 2.3 μM, respectively. SC-45662 and SC-41661A also inhibited the synthesis of 5-HETE from [^{14}C]AA by ionophore-stimulated human PMNs with IC_{50} values of 68 and 17 μM, respectively (Table 1).

Table 1. Inhibition of arachidonate 5-lipoxygenase by SC-45662 and SC-41661A

Enzyme	IC_{50} (μM)	
	SC-45662	SC-41661A
Cell-free RBL-1 5-LO	3.5 ± 0.4	0.3 ± 0.1
Intact RBL-1 5-LO	11.4 ± 5	2.3 ± 1
Intact human PMN 5-LO	68 ± 2	17 ± 4
Human platelet 12-LO	>100	>100
Soybean 15-LO	>100	>100
Ram seminal vesicle CO	>100	>100
Hog pancreatic PLA_2	>100	>100

Assay methods are described in Materials and Methods. Values are means ± SD of four separate experiments.

* A known enzyme inhibitor was included in each experiment. The following IC_{50} values (μM) were determined (mean ± SD, N = 4): cell-free 5-LO – diphenyldisulfide (0.5 ± 0.02); intact cell 5-LO – nordihydroguaiaretic acid (NDGA) (0.7 ± 0.3); PMN 5-LO – NDGA (1.0 ± 0.3); 12-LO – NDGA (0.008 ± 0.001); 15-LO – NDGA (3.2 ± 0.3); CO – indomethacin (0.3 ± 0.1); PLA_2 – mepacrine (130 ± 30).

Effects of SC-45662 and SC-41661A on other positional arachidonate lipoxygenases and phospholipase A_2. SC-45662 showed little or no inhibitory activity (<30%) towards ovine seminal vesicle CO, human platelet 12-LO, soybean 15-LO or hog pancreatic PLA_2 at concentrations up through 100 μM (Table 1). SC-41661A, at 100 μM, inhibited CO 44% and 12-LO 33%. Little or no inhibition (<30%) of 15-LO or PLA_2 was observed with SC-41661A through 100 μM.

Inhibitory effect of SC-45662 on primed and unprimed superoxide generation from human PMNs. Superoxide generation by freshly isolated human PMNs was quantified by superoxide dismutase inhibitable reduction of ferricytochrome *c*. SC-45662 inhibited superoxide generation stimulated with the bacterial chemotactic peptide fMLP or human recombinant C5a in a concentration responsive manner (Fig. 2A). The IC_{50} values with fMLP and C5a were 12 and 5 μM, respectively. Bacterial LPS, TNF-α and PMA at concentrations that do not induce superoxide release were found to prime neutrophils for enhanced generation of superoxide when subsequently challenged with fMLP or C5a (Fig. 3), as has been reported previously [25, 29, 30]. The degree of priming varied with PMN preparations from different donors. SC-45662 was found to inhibit these primed responses with IC_{50} values between 5 and 15 μM, depending upon the donor (Fig. 4, A and B). Superoxide release induced by PMA and serum-activated zymosan (0.75 mg/mL) was not inhibited by SC-45662 at doses up to 100 μM (data not shown).

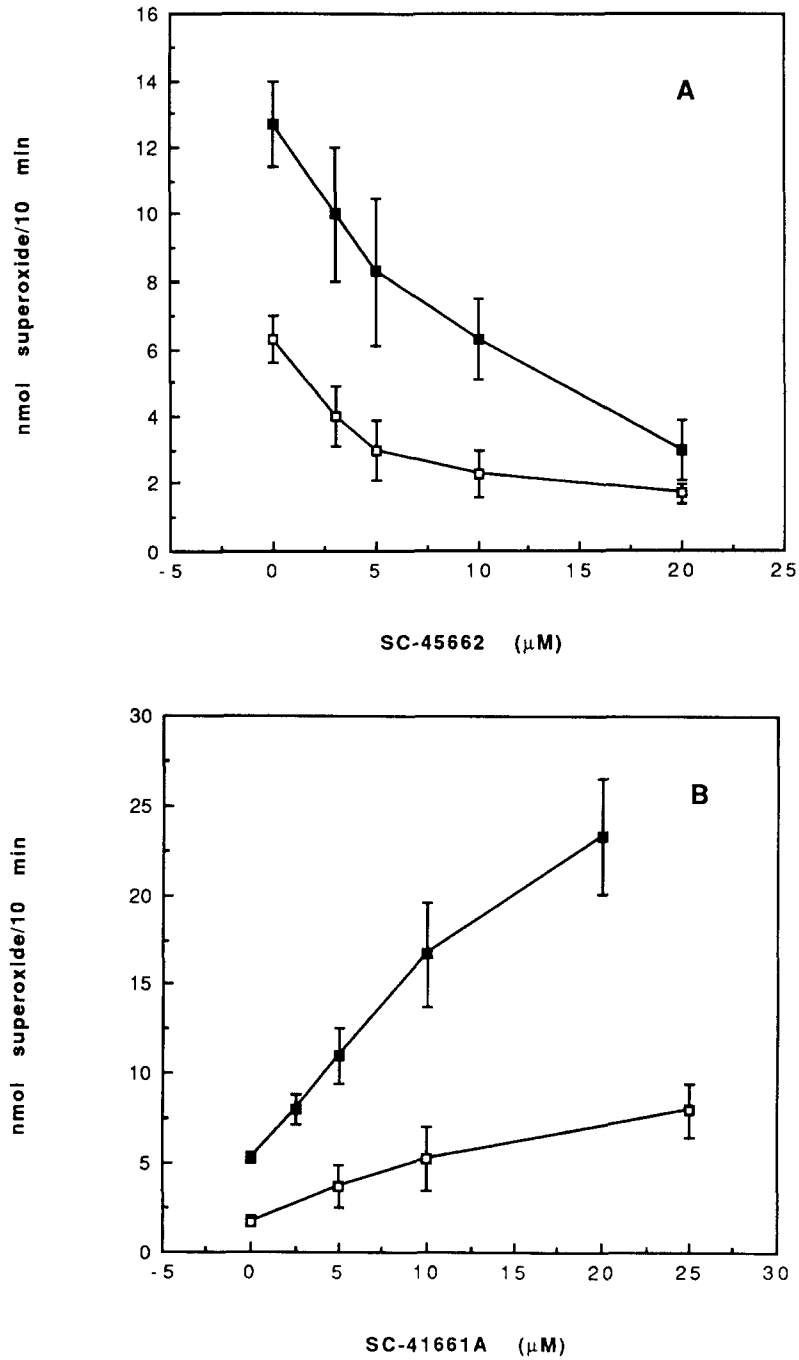


Fig. 2. Effects of SC-45662 and SC-41661A on human PMN superoxide generation. Human PMNs (2×10^6) were preincubated with SC-45662 (A) or SC-41661A (B) for 5 min at 37° in the presence of 50 μM ferricytochrome *c* and stimulated with 0.1 μM fMLP (■) or 10 nM C5a (□). The total superoxide released after 10 min was quantified by the superoxide dismutase inhibitable reduction of ferricytochrome *c*, as measured by increased absorbance at 550 nm. Data shown are the means ± SD from three separate experiments using different blood donors in each experiment.

Stimulatory effect of SC-41661A on primed and unprimed superoxide generation from human PMNs. SC-41661A, in contrast to SC-45662, did not inhibit superoxide but was found to prime PMNs for superoxide production when subsequently challenged with fMLP or C5a (Fig. 2B). SC-41661A by itself, at concentrations up through 100 μM, did not induce superoxide release. When cells were incubated with SC-41661A in combination with the priming agents LPS, TNF-α or PMA and then stimulated with fMLP

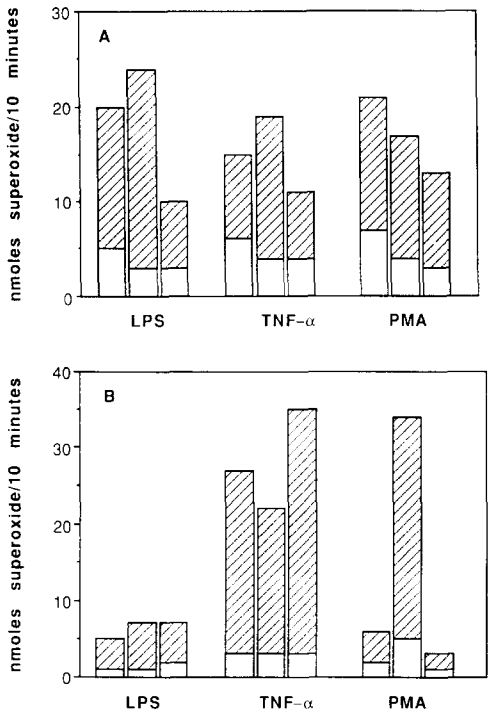


Fig. 3. Priming effect of lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α) and phorbol myristate acetate (PMA) on fMLP and C5a induced superoxide generation. PMNs (2×10^6) were preincubated with LPS (50 ng/mL, 30 min), TNF-α (500 U/mL, 10 min) or PMA (5 nM, 10 min) before inducing superoxide release by the addition of 0.1 μM fMLP (A) or 10 nM C5a (B). The total superoxide released in primed (▨) and unprimed (□) cells was quantified after 10 min. Each bar is data from a single blood donor, representative of several experiments.

or C5a, there was an increase in superoxide production over that obtained in the absence of SC-41661A (Fig. 4, C and D). With C5a, the priming effect of SC-41661A reached a maximum at approximately 25 μM in the presence of LPS and TNF-α and then appeared to decline at higher concentrations of SC-41661A. The generation of superoxide induced either by higher concentrations of PMA (40 nM) or by serum-activated zymosan (0.75 mg/mL) was not enhanced by SC-41661A at concentrations up through 100 μM (data not shown).

Reversibility of the modulatory effects of SC-45662 and SC-41661A on fMLP-induced superoxide generation by human PMNs. The inhibitory effect of SC-45662 and the stimulatory effect of SC-41661A on fMLP-induced superoxide generation were readily reversible by washing the cells and resuspending them in fresh medium. PMNs were preincubated with vehicle, 15 μM SC-45662 or 15 μM SC-41661A for 10 min at 37°. The cells were then washed by centrifugation at 200 *g* for 5 min followed by resuspension in fresh buffer one or two times prior to inducing superoxide generation with fMLP. Incubation of PMNs with 15 μM SC-45662 resulted in a 100% inhibition of superoxide release. This inhibition was decreased to 36% after one wash and 15% after a second wash. The 182% stimulation of superoxide generation by 15 μM SC-41661A was reduced to 8% after one wash and 0% after two washes.

Interaction of SC-45662 and SC-41661A with the fMLP receptor. Regulation of fMLP-stimulated activity could be explained by interactions at specific cell surface receptors. To investigate this possibility, SC-45662, SC-41661A or unlabeled fMLP was incubated with 2×10^6 PMNs for 10 min prior to the addition of 20 nM [^{3}H]fMLP. After an additional 30 min, the incubation was terminated by the addition of cold buffer and centrifugation. The radioactivity remaining in the cell pellet was then determined. Under the study conditions, 50% of the bound [^{3}H]fMLP was blocked by 50 nM unlabeled fMLP (Fig. 5). SC-45662 inhibited [^{3}H]fMLP binding 50% at a concentration of 50 μM. SC-41661A did not block fMLP binding at as high a dose as 100 μM. The apparent 20–30% increase in fMLP binding at 10–100 μM concentrations of SC-41661A was not concentration responsive.

Effects of SC-45662 and SC-41661A on superoxide dismutase and catalase activities. SC-45662 and SC-41661A at concentrations through 100 μM had little (<25%) or no inhibitory effect on the reduction of ferricytochrome *c* by superoxide generated in the xanthine-xanthine oxidase reaction, nor did the compounds interfere with the superoxide dismutase scavenging of superoxide in a concentration-related manner (data not shown). The compounds did not suppress catalase-catalyzed cleavage of hydrogen peroxide to oxygen at concentrations as high as 100 μM (data not shown).

DISCUSSION

SC-45662 and SC-41661A are two 5-LO inhibitors with little or no effect on other enzymes that metabolize arachidonic acid such as 12-LO, 15-LO and CO. Although both compounds are derived from the same chemical series and have similar 5-LO inhibitory activities, they have opposite effects on superoxide generation in purified human PMNs.

SC-45662 inhibited 5-HETE production by isolated RBL-1 cell-derived 5-LO and LTC_4 production by intact RBL-1 cells with IC_{50} values of 3.5 and 11.4 μM, respectively. SC-45662 at 2–20 μM concentrations also effectively blocked the generation of superoxide induced by the peptidyl chemotactic agents fMLP and C5a. SC-41661A was more potent as a 5-LO inhibitor with IC_{50} values of 0.3 and 2.3 μM against the isolated enzyme and intact cells, respectively. However, in contrast to SC-45662, SC-41661A stimulated superoxide generation induced by fMLP and C5a 2 to 4-fold at concentrations of 5–50 μM.

SC-41661A alone did not induce superoxide generation but acted as a priming agent for subsequent induction by fMLP or C5a. Furthermore, SC-45662 and SC-41661A were able to decrease or increase, respectively, superoxide production in PMNs primed to release increased amounts of superoxide with agents such as LPS, TNF-α and PMA. In fact, all the primed PMN systems we

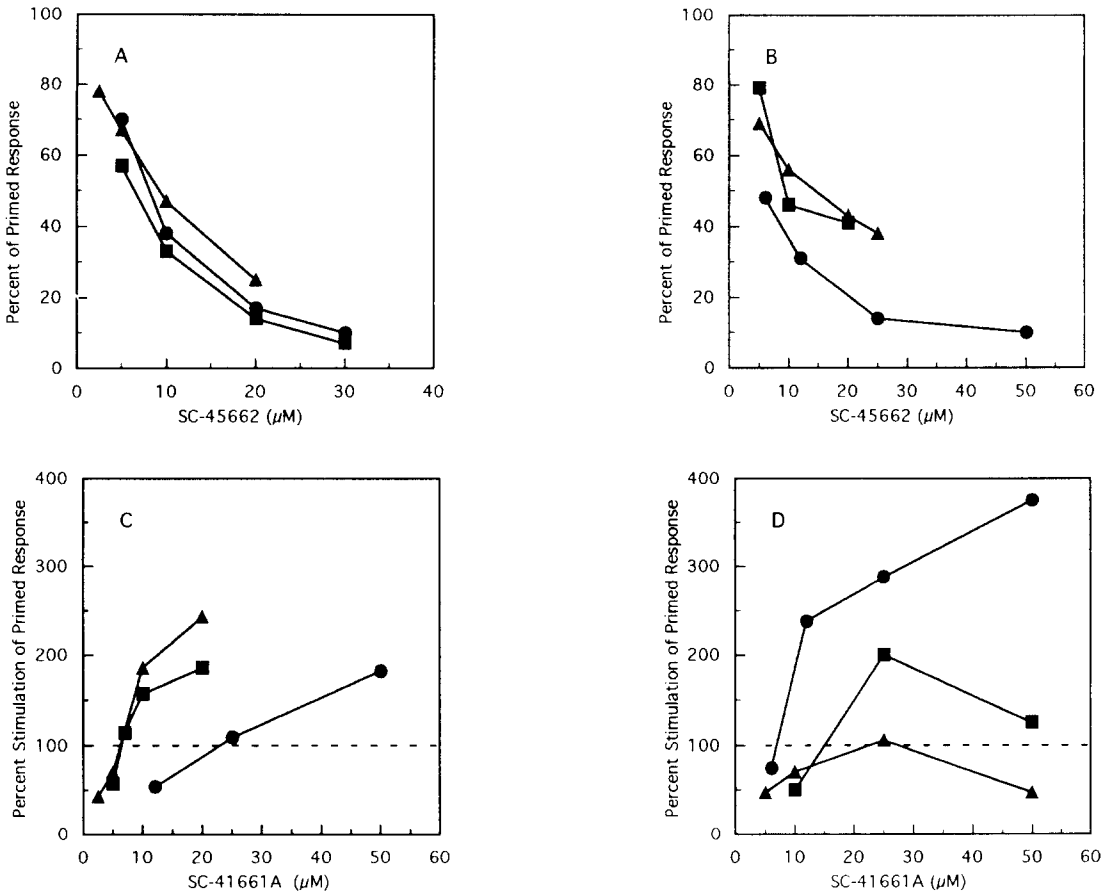


Fig. 4. Effects of SC-45662 and SC-41661A on primed superoxide release. PMNs (2×10^6) were primed with 50 ng/mL LPS (■), 500 U/mL TNF-α (▲) or 5 nM PMA (●) before adding SC-45662 and 0.1 μM fMLP (A) or 10 nM C5a (B). Per cent of primed response = 100 − [(primed compound treated/primed) × 100]. Similarly, PMNs were primed before adding SC-41661A and 0.1 μM fMLP (C) or 10 nM C5a (D). Per cent stimulation of primed response = −[1-(primed compound treated/primed) × 100]. Each line represents data averaged from two blood donors (total N = 24). The dashed line represents a doubling of superoxide generation.

studied, which also included platelet-activating factor and substance P primed cells, were inhibited by SC-45662 and stimulated by SC-41661A when triggered to release superoxide by either fMLP or C5a.

The regulatory effects of SC-45662 and SC-41661A on superoxide generation are clearly independent of their arachidonate 5-LO inhibitory properties. LY-233569, a selective antagonist of 5-LO activity, did not block fMLP-induced superoxide production at a concentration 100 times higher than the IC_{50} reported for blocking LTB_4 production in human PMNs [31]. E-5110, a free radical scavenger with both 5-LO and CO inhibitory activities, was found to block human PMN superoxide generation at approximately 8-fold greater concentrations than those required to block LTB_4 production by the same cell type [32]. MK-886, an inhibitor of the 5-LO activating protein, FLAP, with no direct effect on 5-LO, did not reduce superoxide production significantly in intact PMNs at a concentration approximately 100-fold higher than that required to reduce LTB_4 production [33]. Indomethacin, a non-steroidal anti-inflammatory drug which is a potent inhibitor of cyclooxygenase, caused only a small increase in superoxide production at concentrations that greatly inhibit cyclooxygenase activity [34]. Other non-steroidal anti-inflammatory drugs, which included piroxicam, ibuprofen, aspirin and sulindac, had no effect on superoxide generation by PMNs. Our data along with these examples, clearly support our hypothesis that the metabolism of arachidonate by 5-LO, 12-LO, 15-LO or CO and the generation of superoxide, although induced by similar stimuli, are independently regulated.

SC-45662 and SC-41661A both contain the same 2,6-di-*tert*-butyl-4-thiaphenol reducing, anti-oxidant functional group. Both compounds were easily oxidized to either their sulfone or sulfoxide derivatives with hypohalite or peroxide reagents in the organic chemist's laboratory using pure regents and organic solvents. In addition, SC-45662 did not react chemically with potassium superoxide in DMSO or dimethoxyethane with or without 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane,

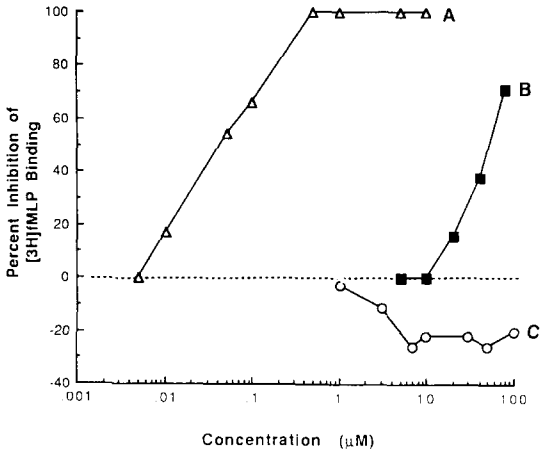


Fig. 5. Effects of SC-45662 and SC-41661A on [^{3}H]fMLP binding to human PMNs. Human PMNs (2×10^6) were preincubated with unlabeled fMLP (A), SC-45662 (B) or SC-41661A (C) for 10 min at room temperature. [^{3}H]fMLP (20 nM) was added, and the incubation was continued for 30 min. Binding was stopped with the addition of ice-cold buffer and centrifugation. Supernatants were removed, the cell pellets were counted, and per cent inhibition of [^{3}H]-fMLP binding relative to the control sample was determined. Each line represents data from a single blood donor, representative of several experiments. The dashed line represents the baseline.

Aldrich) added to enhance solubility at room temperature. Comparison of the biochemical, antioxidant properties of the two compounds reported here illustrates that the non-antioxidant portion of the molecule is critical for both the biochemical potency and selectivity profiles.

Superoxide generation induced directly by PMA or serum-activated zymosan was unaffected by either SC-45662 or SC-41661A at concentrations as high as 100 μM. The data with SC-45662 are in contrast to that obtained with sheep granulocytes where the compound blocked opsonized zymosan particle-induced superoxide production with an approximate IC_{50} of 81 μM [35]. This may be due to differences in cell types or zymosan preparations. SC-45662 did not interfere with superoxide dismutase scavenging of superoxide nor did it suppress catalase-catalyzed cleavage of hydrogen peroxide to oxygen. Also, the lack of effect of SC-45662 on superoxide generation by the xanthine/xanthine oxidase system indicates that the compound does not interfere with the mechanism of superoxide generation nor does it scavenge superoxide. These data suggest that SC-45662 neither affects intracellular enzymes, which metabolize superoxide and other reactive oxygen species, nor the substrates or products of these enzymes. The lack of effect of SC-45662 on superoxide generation induced by PMA would indicate that SC-45662 interferes with a step prior to activation of protein kinase C in the receptor-dependent mechanism of signal transduction, which leads to phosphorylation of NADPH-oxidase and the production of superoxide [11]. Its effect on superoxide generation was readily reversible by simply washing the cells. SC-45662 partially inhibited the binding of radiolabeled fMLP to PMN membrane receptors (Fig. 5). It is unlikely that receptor binding antagonism completely explains SC-45662 suppression of superoxide release because inhibition of superoxide generation occurs at lower concentrations than those required to compete for fMLP binding.

SC-41661A may also affect an early step in the activation of NADPH-oxidase since SC-41661A did not affect superoxide generation induced by PMA. As with SC-45662, the effects on fMLP-induced superoxide generation by SC-41661A were also reversible. Increased binding of fMLP to its receptor did not appear to be a potential mechanism in this case, since SC-41661A did not affect receptor binding in a concentration-dependent manner. SC-41661A did not affect xanthine oxidase, catalase or superoxide dismutase activity.

The biological consequences of altering superoxide generation *in vivo* remain unknown. Priming for oxygen radical generation by agents such as LPS, TNF-α, PMA, platelet-activating factor and substance P would suggest that the pro-inflammatory properties associated with these agents may be related, in part, to their amplification of radical generation. Our finding that SC-45662 can suppress the generation of superoxide and presumably other oxygen radicals derived from superoxide even in primed systems would suggest that the compound may play a protective role against oxidative damage in addition to its anti-inflammatory 5-LO inhibitory property. In support of this hypothesis, SC-45662 was found active in the rat adjuvant-induced arthritis model and in the guinea pig antigen-induced bronchoconstriction model [36]. Conversely, the generation of reactive oxygen species by PMNs can be beneficial. Cells deprived of oxygen phagocytize but do not kill microbes as efficiently as in the presence of oxygen [37]. Failure to produce reactive oxygen species results in chronic granulomatous disease characterized by persistent bacterial infections [37, 38]. A compound that enhances the generation of oxygen metabolites by PMNs could increase PMN mediated host defense. For example, there is evidence for the involvement of activated oxygen species derived from PMNs and macrophage in the TNF-α-induced killing of tumor cells [39]. SC-41661A was effective *in vivo* in preventing the formation of ovarian tumors in an ovarian cancer cell model and in decreasing the formation of melanotic lung lesions in a lung melanoma metastasis model [40, 41]. In a similar manner, this compound may also be beneficial in the treatment of various infectious diseases.

In summary, we have shown that SC-45662 and SC-41661A are selective arachidonate 5-LO inhibitors with markedly opposite effects on human PMN superoxide release and that their biochemical selectivity profiles are not exclusively dependent on their expected antioxidant properties. The efficacy of these compounds and the biological consequences of altering superoxide generation and 5-LO activity *in vivo* remain to be investigated. These new

compounds will provide excellent tools in support of these investigations.

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