Pharmacological Characterization of SC-57461A (3-[Methyl][3-[4-(phenylmethyl)phenoxy]propyl]amino]propanoic Acid HCl), a Potent and Selective Inhibitor of Leukotriene A₄ Hydrolase I: In Vitro Studies

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ABSTRACT
Leukotriene (LT) B₄ is an inflammatory mediator that has been implicated in the pathogenesis of various diseases, including inflammatory bowel disease and psoriasis. As the rate-limiting step for LTB₄ production, LTA₄ hydrolase represents an attractive target for therapeutic agents that interfere with LTB₄ production. In the present study we evaluated a chemically novel compound designated SC-57461A (3-[methyl][3-[4-(phenylmethyl)phenoxy]propyl]amino]propanoic acid HCl) as an inhibitor of LTA₄ hydrolase. Pharmacological comparisons are made to its free acid SC-57461. SC-57461A is a potent competitive inhibitor of recombinant human LTA₄ hydrolase when either LTA₄ (IC₅₀ = 2.5 nM, Kᵢ = 23 nM) or peptide substrates (IC₅₀ = 27 nM) are used. In human whole blood, the IC₅₀ for calcium ionophore-induced LTB₄ production was 49 nM, indicative of good cell penetration. Whole blood production of the cyclooxygenase metabolite thromboxane B₂ was not affected. SC-57461A was also active in several other species, including mouse, rat, dog, and rhesus monkey. The data indicate that SC-57461A is a potent and selective inhibitor of LTA₄ hydrolase.

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ABBREVIATIONS:
LT, leukotriene; SC-57461A, 3-[methyl][3-[4-(phenylmethyl)phenoxy]propyl]amino]propanoic acid HCl; LTA₄, 5(S)-trans-5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid; LTB₄, 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; HLTA₄, recombinant human leukotriene A₄ hydrolase; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; RP-HPLC, reversed phase-high-performance liquid chromatography; pNA, p-nitroanilide; TxB₂, thromboxane B₂; A23187, calcium ionophore A23187 (Calcimycin).
A series of nonpeptide transition-state analog inhibitors of LTA4 hydrolase were designed on the basis of their ability to inhibit the aminopeptidase activity (Yuan et al., 1993), and more recently a series of inhibitors that incorporate potential zinc-chelating moieties have been described (Hogg et al., 1995). Some of these inhibitors display time-dependent kinetics and most of them preferentially inhibit the peptidase activity over the epoxide hydrolase activity (Penning et al., 1995; Hogg et al., 1998). The hydroxamic acid-containing inhibitor-kinetics was determined using an RP-HPLC assay to quantify LTB4. The assay contained 1 μg of enzyme (143 nM final concentration) in 100 μl of 50 mM HEPES buffer, pH 8.0, containing 1 mg/ml fatty acid-free BSA. The reaction was initiated by the addition of 25 μM LTA4 free acid in ethanol (to a final ethanol concentration of 2%) and incubated at 25°C for 15 to 30 s. The reaction was stopped by the addition of 2 volumes of cold buffer (3:1, 200 mM citrate, pH 3.5/0.1% acetic acid), 40% buffer A (60% acetonitrile, 40% methanol, 0.1% acetic acid), 40% buffer B (0.1% acetic acid in water), and eluted over 20 min with a concave gradient to 90% buffer A, 10% buffer B. The eluate was monitored at 270 nm, and the LTB4 quantified using an in-house generated anti-LTB4 polyclonal antibody, R11 (Gierse et al., 1993).

**Peptidase Assay.** Aminopeptidase activity was determined spectrophotometrically by the release of the colorimetric product 4-nitroaniline from L-arginine-p-nitroanilide (Arg-pNA) or L-arginine-p-nitroanilide (Arg-pNA) in 50 mM Tris-HCl, pH 7.5, containing 1 mg/ml fatty acid-free BSA in 200 μl. The reaction was started by the addition of recombinant human LTA4 hydrolase. Absorbance was continuously monitored at 405 nm and the reaction allowed to proceed at room temperature. For the kinetic determinations, the substrate was varied from 0.083 mM to 1 mM, and 50 ng of protein (3.6 nM final concentration) was used with a total reaction time of 60 min. Inhibition kinetic constants (Kic values) were calculated using the program k-cat by BioMettalics (Princeton, NJ) or by the graphical method of Dixon (1972).

**Epoxyde Hydrolase Assay.** Recombinant human cytosolic epoxide hydrolase was assayed using LTA4 as the substrate. The assay was carried out in a microplate reader using a commercial enzyme-linked immunosorbant assay (ELISA).

**Fig. 1.** Reaction catalyzed by LTA4 hydrolase.

**Fig. 2.** Structure of SC-57461A (3-[methyl][3-[4-(phenylmethyl)phenoxyl]propyl]amino]propanoic acid HCl).
was conducted as described for the LTA₄ hydrolase kinetic assay except the reaction was allowed to proceed for 1 min before quenching. 5(S),6(R)-Dihydroxyeicosatetraenoic acid was quantitated by RP-HPLC exactly as described for LTB₄.

Assays for Arachidonic Acid-Metabolizing Enzymes. Assays for other enzymes known to metabolize arachidonic acid into inflammatory mediators were performed as previously described. These include human recombinant 5-lipoxygenase (Smith et al., 1995), human recombinant cyclooxygenase-1 and cyclooxygenase-2 (Gierse et al., 1995), and leukotriene C₄ synthase activity in THP-1 cells (Welsch et al., 1994).

Whole Blood Eicosanoid Production. Human blood collected in heparin containing Vacutainer tubes was diluted 1:4 with RPMI-1640 (Gibco, Carlsbad, CA) and 200 µl was added per well in 96-well microtiter plates. Compounds diluted in 1% DMSO were added to the blood in duplicate and allowed to incubate for 15 min at 37°C in a humidified incubator (5% CO₂). Calcium ionophore A23187 (Cayman Chemical) and 653 calcimycin (20 µg/ml, 1% DMSO final concentration) was added and the incubation continued for 10 min. The incubation was terminated by centrifugation (600 × g, 10 min, 4°C). Supernatants were analyzed for LTB₄ and TxB₂ with ELISA (human recombinant cyclooxygenase-1 and cyclooxygenase-2 at 100 nM (mouse) or 30 min (mouse, rat, dog). Incubations were terminated by centrifugation as previously described for human whole blood. Supernatants were analyzed for LTB₄ and TxB₂ with ELISA by using commercially available reagents (Cayman Chemical) and the in-house-generated anti-LTB₄ polyclonal antibody R11.

Heparinized blood from other species (rhesus monkey, rat, mouse, and dog) was diluted 1:1 with RPMI-1640 immediately before aliquoting 200 µl/well into microtiter plates. Preincubations with inhibitor for 15 min. Experimental conditions were optimized for each species in preliminary experiments. Calcium ionophore A23187 concentrations (µg/ml) were 10 for mouse and rat, and 20 for mouse and dog. Incubations were continued after A23187 addition for 20 min (mouse) or 30 min (monkey, rat, dog). Incubations were terminated by centrifugation as previously described for human whole blood. Supernatants were analyzed for LTB₄ and TxB₂ with ELISA by using in-house-generated anti-LTB₄ antibody and/or commercially available reagents (Cayman Chemical).

Materials. SC-57461 and SC-57461A (>95% purity), zileuton [N-(1-benzo[b]thien-2-yl)ethylnitro-N-hydroxyurea], and A-78773 [N-[3-[5-(4-fluorophenoxo)-2-furanyl]-1-methyl-2-propynyl]-N-hydroxyurea] were synthesized by the Chemistry Department at Pharmacia Research and Development, Skokie, IL.

Enzymes. Purified enzymes from nonhuman species were cloned, expressed, and purified as described for the human. Clones were obtained from a mouse lung cDNA library for the murine, and a rat spleen cDNA library for the rat. After purification, the specific activities of the epoxide hydrolase were 420 nmol/min/mg for the murine (>95% by SDS-polyacrylamide gel electrophoresis), and 653 nmol/min/mg for the rat (89% pure by SDS-polyacrylamide gel electrophoresis). Recombinant human cytosolic epoxide hydrolase was a generous gift from Bruce D. Hammock (Department of Entomology, University of California, Davis, CA) (Beetham et al., 1993).

Results

Inhibition of rhLTA₄ Hydrolase. SC-57461A and SC-57461 are both very potent, nonpeptide inhibitors of rhLTA₄ hydrolase when either LTA₄ or peptide substrates are used (Table 1). No inhibition of rhLTA₄ hydrolase was observed with either the first- (zileuton) or second-generation (A-78773) 5-LO inhibitors (Carter et al., 1991; Harris et al., 1995), demonstrating selectivity of these inhibitors for the 5-LO enzyme. In contrast to previously described LTA₄ hydrolase inhibitors (Wettermol et al., 1995; Hogg et al., 1998), SC-57461A is equipotent as an inhibitor of the hydrolase and peptide activity. SC-57461A is equipotent with kelatorphan as an inhibitor of LTA₄ hydrolase (Penning et al., 1995).

SC-57461 displayed competitive kinetics when either LTA₄ (Fig. 3) or a peptide substrate is used (data not shown) (Table 1). These assays are routinely run after short-term preincubation of the enzyme and inhibitor. Therefore, the time-dependent inhibition of SC-57461 was also investigated. Inhibition was measured with no preincubation (enzyme added last) or defined intervals of preincubation. As illustrated in Fig. 4, no change in the inhibition of LTA₄ hydrolase was seen after preincubation of the enzyme with inhibitor from 0 to 5 min. Further studies extended the preincubation time scale to 24 h without a significant change in the IC₅₀ (data not shown).

SC-57461 and SC-57461A were also tested as inhibitors of purified LTA₄ hydrolase from mouse and rat (Table 2). In these preparations, SC-57461 was equipotent on the human and murine enzymes, but from 2- to 10-fold less active against the rat enzyme.

Specificity of Enzyme Inhibition. SC-57461 was tested for its specificity to inhibit other enzymes in the arachidonic acid cascade. It did not inhibit recombinant human 5-lipoxygenase, recombinant human cyclooxygenase-1, or recombinant human cyclooxygenase-2 at 100 µM or LTA₄ synthase activity in THP-1 cells at 100 µM (data not shown). SC-57461 was also tested as an inhibitor of recombinant human cytosolic epoxide hydrolase (Haeggström et al., 1986; Beetham et al., 1993). By using LTA₄ as the substrate, the IC₅₀ was determined to be 300 µM (Fig. 5). This >1000-fold selectivity of SC-57461 for LTA₄ hydrolase over an unrelated cytosolic epoxide hydrolase is further demonstration of its specificity. In addition, SC-57461 at 100 µM did not inhibit other met-

### TABLE 1

Inhibition values for rhLTA₄ hydrolase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>LTA₄</th>
<th>Arg-pNA</th>
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<tbody>
<tr>
<td></td>
<td>IC₅₀</td>
<td>Kᵢ</td>
</tr>
<tr>
<td></td>
<td>nM</td>
<td></td>
</tr>
<tr>
<td>SC-57461</td>
<td>10 ± 0 (3)</td>
<td>23 (2)</td>
</tr>
<tr>
<td>SC-57461A</td>
<td>2.5 ± 0.05 (4)</td>
<td>NT</td>
</tr>
<tr>
<td>A-78773</td>
<td>&gt;3000</td>
<td>NT</td>
</tr>
<tr>
<td>Zileuton</td>
<td>&gt;100,000</td>
<td>NT</td>
</tr>
<tr>
<td>Kelatorphan</td>
<td>8 (2)</td>
<td>18 (2)</td>
</tr>
</tbody>
</table>

NT, not tested.

a Leucine-pNA as substrate.
alloproteases, including human leukocyte elastase, human liver cathepsin B, rabbit lung angiotensin-converting enzyme or bovine spleen cathepsin D (data not shown). A structurally related LTA₄ hydrolase inhibitor, SC-54581, did not inhibit neutral endopeptidase at 100 nM (data not shown).

Inhibition of Whole Blood Eicosanoid Production.
The activity and selectivity of SC-57461A and SC-57461 were also demonstrated in whole blood under conditions in which both the 5-LO and cyclooxygenase pathways were stimulated simultaneously with calcium ionophore. SC-57461A and SC-57461 were potent inhibitors of LTB₄ production in whole blood from a variety of species (Table 3). Both compounds (IC₅₀ values in human blood, 49 and 65 nM, respectively) are similar in potency to A-78773 in human and rhesus blood. SC-57461A is less potent than A-78773 in the mouse, rat, and dog. In contrast to the purified enzyme, SC-57461 was 3-fold less potent on murine blood compared with human, but the rat/human ratio of activity was similar.

In all species tested, neither SC-57461A nor SC-57461 inhibited the synthesis of TxB₂ (IC₅₀ > 10 μM), a major cyclooxygenase product formed in whole blood under these conditions (data not shown).

SC-57461A did not inhibit LTB₄ stimulated chemotaxis of isolated human neutrophils in Boyden chambers over a concentration range of 1 to 100 μM, suggesting it does not block the ability of LTB₄ to activate LTB₄ receptors (data not shown).

**Discussion**

Previous investigators have followed a two-pronged approach in the search for potent LTA₄ hydrolase inhibitors. The first approach consisted of inhibitors based on the LTA₄ substrate. Djuric et al. (1992) synthesized compounds based on an oxabicycloheptene nucleus in an attempt to mimic the vinyl epoxide of the natural substrate. These compounds did not inhibit the isolated enzyme but inhibited LTB₄ biosynthesis in the HL-60 cell line. Labaudinière et al. (1992) synthesized a series of ω-arylalkanoic acid derivatives in an attempt to mimic the allylic epoxide of LTA₄. The most potent

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>IC₅₀ (nM) ± S.E.</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SC-57461</td>
</tr>
<tr>
<td>Human</td>
<td>10 ± 0 (3)</td>
</tr>
<tr>
<td>Mouse</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Rat</td>
<td>19 ± 7 (3)</td>
</tr>
</tbody>
</table>

Fig. 3. Competitive inhibition of rhLTA₄ hydrolase by SC-57461. One microgram of rhLTA₄ hydrolase was incubated with increasing amounts of SC-57461 (○, 0 nM; △, 50 nM; ▽, 100 nM; ■, 200 nM; ■, 300 nM; △, 400 nM) as described under Experimental Procedures. The inhibition constant derived from the graphical method of Dixon for SC-57461 was Kᵢ = 23 nM.

Fig. 4. Time-independent inhibition of rhLTA₄ hydrolase by SC-57461A. rhLTA₄ hydrolase was preincubated with varying concentrations of inhibitor for the indicated periods of time. LTA₄ was added to start the assay or the enzyme was added last for time 0, and the reaction quenched as described under Experimental Procedures. LTB₄ was quantified by RP-HPLC. Preincubation times: ■, 0 min; ○, 30 s; and △, 5 min.

Fig. 5. Specificity of inhibition of SC-57461 on LTA₄ hydrolase versus cytosolic epoxide hydrolase. Enzyme used: ○, recombinant human cytosolic epoxide hydrolase, IC₅₀ = 300 μM; ●, recombinant human LTA₄ hydrolase, IC₅₀ = 0.10 μM.
of these analogs displayed IC\textsubscript{50} values in the low micromolar range against porcine leukocyte LTA\textsubscript{4} hydrolase.

A second approach focused on the peptide isostere after the discovery that LTA\textsubscript{4} hydrolase is a zinc-containing enzyme displaying aminopeptidase activity. Two series of compounds evolved from the backbones of bestatin and captopril (Yuan et al., 1991, 1992, 1993). In the first series, the investigators used the norstatine backbone of bestatin to build potential peptide transition state analogs. This series eventually evolved to incorporate \(\alpha\)-keto-\(\beta\)- amino esters, which in an aqueous environment hydrated to the gem diol, capable of coordinating with the \(\text{Zn}^{2+}\) in the active site. The most potent of these compounds were shown to be relatively selective for the aminopeptidase activity of LTA\textsubscript{4} hydrolase.

Other zinc binding motifs have also been explored as LTA\textsubscript{4} hydrolase inhibitors. Hydroxamic acids have been shown to be potent inhibitors of LTA\textsubscript{4} hydrolase. Hogg et al. (1995, 1998) reported a series of inverted hydroxamates to be low nanomolar inhibitors of the aminopeptidase activity, but less potent for the epoxide hydrolase activity. Kelatorphan has been shown to be equipotent as an inhibitor of both of the LTA\textsubscript{4} hydrolase activities, but it is not selective for LTA\textsubscript{4} hydrolase (Penning et al., 1995). A very potent \(\beta\)-mercaptotamine (Yuan et al., 1993; Ollmann et al., 1995) has been described, but it also inhibits thromboxane B\textsubscript{2} production in human whole blood. Again, it is assumed that part of its inhibitory activity is due to the zinc-thiol interaction.

SC-57461A is the first reported compound with a structure unrelated to the natural substrate LTA\textsubscript{4} or a peptide isostere to possess potent inhibitory activity against LTA\textsubscript{4} hydrolase. Its IC\textsubscript{50} of 2.5 nM for the epoxide hydrolase activity makes it one of the most potent inhibitors reported to date. It is at least 50-fold more potent against the epoxide hydrolase activity than the most potent inhibitors reported by Yuan et al. (1993), Hogg et al. (1995, 1998), and Labaudinier et al. (1992); as well as captopril and bestatin (Orning et al., 1991a,b; Baker et al., 1995). It is also unique in that its inhibitory activity is due to the zinc-thiol interaction.

### Table 3

**Inhibition of ionophore-stimulated LTB\textsubscript{4} production in whole blood**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human (IC\textsubscript{50} (nM) ± S.E.)</th>
<th>Rhesus</th>
<th>Mouse (IC\textsubscript{50} (nM) ± S.E.)</th>
<th>Rat (IC\textsubscript{50} (nM) ± S.E.)</th>
<th>Dog (IC\textsubscript{50} (nM) ± S.E.)</th>
</tr>
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<tbody>
<tr>
<td>SC-57461</td>
<td>59 ± 16 (4)</td>
<td>108 ± 43 (3)</td>
<td>171 ± 44 (3)</td>
<td>547 ± 238 (3)</td>
<td>240 ± 4.61 (3)</td>
</tr>
<tr>
<td>SC-57461A</td>
<td>49 ± 5 (4)</td>
<td>NT</td>
<td>166 ± 35.3 (3)</td>
<td>466 ± 184 (3)</td>
<td>NT</td>
</tr>
<tr>
<td>A-78773 (5-LO)</td>
<td>79 ± 22.5 (3)</td>
<td>118 ± 33.5 (3)</td>
<td>67 ± 12.1 (3)</td>
<td>50 (2)</td>
<td>57 ± 1.2 (3)</td>
</tr>
<tr>
<td>Zileuton (5-LO)</td>
<td>812 ± 35 (104)</td>
<td>421 ± 60 (13)</td>
<td>634 ± 39.9 (8)</td>
<td>585 ± 87.8 (3)</td>
<td>715 ± 68.8 (8)</td>
</tr>
</tbody>
</table>

NT, not tested.

### In Vitro Effects of SC-57461A

Multiple isoforms of LTA\textsubscript{4} hydrolase have been reported. Erythrocytes and B-lymphocytes have each been reported to contain a second subtype that distinguished itself by its divergent kinetic properties (McGee and Fitzpatrick, 1985; Orning et al., 1990; Odlander et al., 1991). Bigby et al. (1994) reported that epithelial cell-derived LTA\textsubscript{4} hydrolase present in bronchoalveolar lavage fluid lacked aminopeptidase activity and had unique kinetic properties. They found that the profile of inhibition of epithelial cell-derived LTA\textsubscript{4} hydrolase by certain metalloproteinase inhibitors differed from that of the neutrophil enzyme (Baker et al., 1995). Partial N-terminal sequence determinations of the airway epithelial-derived enzyme failed to show any sequence divergence over the amino terminal 17 amino acid span (Ned Seigel, Pharmacia, personal communication). The recent report of an alternative splice site in the LTA\textsubscript{4} hydrolase gene would support these findings (Jendraschak et al., 1996). The specificity of SC-57461A for the postulated subtype is as yet unclear.

SC-57461A retained good potency in inhibiting LTB\textsubscript{4} production in human and rhesus monkey whole blood. These data suggest that SC-57461A readily penetrates the appropriate cells and interacts with ubiquitously expressed LTA\textsubscript{4} hydrolase. These data also show that SC-57461A is fairly metabolically stable in blood and binding to plasma proteins does not negate efficacy. SC-57461A is slightly less potent in rat and dog whole blood. This may reflect a species-dependent decrease in intrinsic activity against the enzyme as supported by the decrease in potency against the isolated rat enzyme, rather than increased instability in whole blood of different species.

SC-57461 and SC-57461A have also been assessed in several animal models. SC-57461 was shown to be orally active with a pharmacodynamic half-life exceeding 24 h. SC-57461 was shown to inhibit LTB\textsubscript{4} production in a rat model of ionophore-induced peritoneal eicosanoid production, in a rat reversed passive Arthus model, and in an arachidonic acid-induced ear edema model in mice (Kachur et al., 2002). SC-57461 and SC-57461A displayed selective inhibition of LTB\textsubscript{4} in a dose-dependent manner in these models.