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β-Lactams SB 212047 and SB 216754 Are Irreversible, Time-Dependent Inhibitors of Coenzyme A-Independent Transacylase

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ABSTRACT

The enzyme coenzyme A-independent transacylase (CoA-IT) has been demonstrated to be the key mediator of arachidonate remodeling, a process that moves arachidonate into 1-ether-containing phospholipids. Blockade of CoA-IT by reversible inhibitors has been shown to block the release of arachidonate in stimulated neutrophils and inhibit the production of eicosanoids and platelet-activating factor. We describe novel inhibitors of CoA-IT activity that contain a β-lactam nucleus. β-Lactams were investigated as potential mechanism-based inhibitors of CoA-IT on the basis of the expected formation of an acyl-enzyme intermediate complex. Two β-lactams, SB 212047 and SB 216754, were shown to be specific, time-dependent inhibitors of CoA-IT activity (IC_{50} = 6 and 20 μM, respectively, with a 10-min pretreatment time). Extensive washing and dilution could not remove the inhibition, suggesting it was irreversible. In stimulated human monocytes, SB 216754 decreased the production of eicosanoids in a time-dependent manner. In an *in vivo* model of phorbol ester-induced ear inflammation, SB 216754 was able to inhibit indices of both edema and cell infiltration. Taken together, the results support two hypotheses: 1) CoA-IT activity is important for the production of inflammatory lipid mediators in stimulated cells and *in vivo* and 2) the mechanism by which CoA-IT acts to transfer arachidonate is through an acyl-enzyme intermediate.

CoA-IT is an enzyme responsible for the remodeling of arachidonate between different phospholipids (Snyder *et al.*, 1992; Winkler and Chilton, 1995). Specifically, CoA-IT seems to selectively remodel arachidonate and other long-chained, unsaturated fatty acids (Chilton *et al.*, 1983; Kramer and Deykin, 1983; Sugiura *et al.*, 1987; Winkler *et al.*, 1991). Additional studies showed that it is responsible for the remodeling of arachidonate from 1-acyl-containing phospholipids into 1-alkyl- and 1-alkenyl-containing phospholipids (Chilton and Murphy, 1986; Sugiura *et al.*, 1987). To explore the role of CoA-IT in inflammatory cells, we reported previously the characterization of tool compounds that block CoA-IT activity and arachidonate remodeling (Chilton *et al.*, 1995). Using these tools, we demonstrated that this CoA-IT-mediated movement of arachidonate is important for several functions of stimulated inflammatory cells, including the production of PAF, release of free AA, and production of prostanoids (Winkler *et al.*, 1995).

Because of the importance of CoA-IT to inflammatory cell function, it is meaningful to gain insight to its mechanism of action. We hypothesized previously that the enzyme CoA-IT is part of a family of transacylases, typified by lecithin cholesterol acyl-transferase (Winkler and Chilton, 1993). This hypothesis details a catalytic mechanism in which an active site nucleophile in CoA-IT attacks the acyl carbonyl of the *sn*-2 arachidonate of phospholipids, forming a covalent interaction between arachidonate and CoA-IT and liberating lyso phospholipid. The covalently attached arachidonate then can be donated to a suitable lyso phospholipid acceptor. Based on this mechanism, the same CoA-IT nucleophile could attack the ketone within a β-lactam ring, opening the ring and forming a covalent interaction between CoA-IT and the β-lactam-containing compound. Further reaction by decomposition of β-lactams, such as penicillins and cephalosporins, resulting in stable enzyme/product coupling, is well docu-

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ABBREVIATIONS: CoA-IT, coenzyme A-independent transacylase; AA, arachidonic acid; BSA, bovine serum albumin; GPC, *sn*-glycero-3-phosphocholine; 5LO, 5-lipoxygenase; PLA_2, phospholipase A_2; PBS, phosphate-buffered saline; PAF, platelet-activating factor; TLC, thin layer chromatography; HBSS, Hanks' balanced salt solution; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LT, leukotriene; PG, prostaglandin.

mented for a number of enzymes (Brenner and Knowles, 1981; Chabin *et al.*, 1993; Vilain *et al.*, 1993; Green *et al.*, 1995). We examined a number of β-lactams for their ability to inhibit CoA-IT activity and identified some compounds that seem to undergo a mechanism-based reaction as described.

We report here the characterization of two β-lactam inhibitors of CoA-IT activity: SB 212047 [4-methoxybenzyl(3*S*, 4*R*)-6-bromo-6-[(1-methyl-1,2,3-triazol-4-yl)-hydroxymethyl] penicillanate] and SB 216754 [(3*S*,4*R*)-4-[(isobutenyloxy)-3-triphenylmethylamino)]azetidin-2-one]. The data demonstrate that these compounds are time-dependent, selective inhibitors of CoA-IT activity. They seem to interact with CoA-IT in an irreversible manner, supporting the proposed mechanism of action of CoA-IT. In addition, inhibition of CoA-IT by a β-lactam compound reduced the production of PAF and eicosanoids by isolated, stimulated inflammatory cells and blocked indices of inflammation *in vivo*, supporting the notion that CoA-IT activity is critical for inflammatory cell function.

Experimental Procedures

Materials. [^{3}H]Acetic acid, sodium salt (50–100 Ci/mmol), [^{3}H]arachidonate-labeled *Escherichia coli* (10 mCi/mmol, 4–8 nmol P_i/10 μl), and 1-[^{3}H]alkyl-2-lyso-GPC (30–60 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, MA). Histopaque-1077, Percol, and common laboratory chemicals were purchased from Sigma Chemical (St. Louis, MO). 1-Alkyl-2-lyso-GPC was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Silica gel G plates were from Analtech (Newark, DE). Essentially fatty acid-free BSA was obtained from Calbiochem (San Diego, CA). Silica gel columns were from Baker (Phillipsburg, NJ). All fatty acids were from Nu-Chek Prep (Elysian, MN).

Preparation of cells. Neutrophils were prepared from heparinized venous blood collected from healthy donors and isolated according to the procedure of Boyum (1968) using the Histopaque-1077 technique as described previously (Winkler *et al.*, 1993). The final leukocyte preparation was suspended in Dulbecco's PBS (137 mM NaCl, 8.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl; GIBCO, Grand Island, NY) and was >95% viable and pure, as determined by trypan blue exclusion and histological examination. Neutrophils were resuspended at a concentration of 10^7 cells/ml and stimulated with A23187 as described in the figure legends.

Human monocytes were obtained from leukocyte-rich packs (Biological Specialties, Lansdale, PA). The cells were washed twice with HBSS, layered over Histopaque-1077, and then centrifuged at 1000 × *g* for 30 min to obtain a buffy coat enriched in monocytes. These cells were washed with HBSS, resuspended in media (RPMI-1640, 10% fetal bovine serum, 0.2 mM L-glutamine, 2.5 mM HEPES), layered over an equal volume of 46% Percol/54% media, and then centrifuged for 30 min at 1000 × *g*. The monocytes at the interface were collected and washed with HBSS; the resulting monocyte preparation was >85% pure and >95% viable. Monocytes were suspended at 5 × 10^6 cells/ml and stimulated with 1 μM A23187, and supernatant fluids were collected after 15 min.

CoA-IT activity. CoA-IT activity in U937 microsomes was measured as described previously (Winkler *et al.*, 1991) in a total volume of 100 μl. Microsomes (5–20 μg of protein) from a centrifugation at 100,000 × *g* for 60 min (Winkler *et al.*, 1991) were diluted in PBS with 1 mM EGTA to the desired protein concentration. The reaction was initiated by the addition of 1-[^{3}H]alkyl-2-lyso-GPC (0.1 μCi/tube) and 1 μM final cold 1-alkyl-2-lyso-GPC in assay buffer with 0.25 mg/ml fatty acid-poor BSA. The reaction was run for 2–10 min at 37°, times chosen to be on the linear portion of the product production curve. The reaction was stopped, the lipids were extracted (Bligh and Dyer, 1959), and materials from an aliquot of the chloroform extract were separated by TLC in chloroform/methanol/acetic acid/water (50:25:8:4, v/v/v/v; System I, Analtech, Newark, DE) and visualized by radioscanning (Bioscan, Washington, D.C.). The product, 1-[^{3}H]alkyl-2-acyl-GPC, was scraped and quantified by liquid scintillation spectroscopy. With this TLC system, the R_F values for synthetic standards of 1-alkyl-2-lyso-GPC and 1-alkyl-2-acyl-GPC were ~0.24 and ~0.64, respectively.

Enzyme assays. PLA_2 activities were determined using [^{3}H]arachidonate-labeled *Escherichia coli* membranes by measuring the liberation of free [^{3}H]AA (Marshall and McCarte-Roshak, 1992). Human type II, 14-kDa PLA_2 was purified from a clone expressed in Chinese hamster ovary cells (Stadel *et al.*, 1992). Cytosolic 85-kDa PLA_2 (Kramer *et al.*, 1991) was obtained from a clone expressed in baculovirus-infected insect cells (Amegazie *et al.*, 1993). 5LO activity was measured by monitoring O_2 consumption in a reaction mixture of 10 μM AA, 5 μM sonicated dioleoyl phosphatidylcholine, 150 mM NaCl, 5% ethylene glycol, and 0.2 mM $CaCl_2$ (Marshall *et al.*, 1991). 5LO activity was obtained from the cytosolic fraction (100,000 × *g*, 60 min) of RBL-1 cells. Cyclooxygenase activity was measured using the enzyme from ram seminal vesicles, incubated with 50 μM AA (Rabinovici *et al.*, 1993). The reaction was terminated by the addition of 10% trichloroacetic acid, and products were quantified by absorbance at 532 nm. CoA-dependent acylation activity was determined using U937 microsomes that were diluted in Tris buffer (100 mM, pH 7.4) and incubated with 12–15 μM acyl-CoA and 0.015 μCi 1-[^{14}C]-acyl-2-lyso-GPC with 1 μM cold 1-acyl-2-lyso-GPC. After 10 min at 37°, the samples were extracted and developed on TLC plates (System I), and the amount of 1,2-diacyl-GPC product was determined. PLA_2 activities were determined using *E. coli* membranes by measuring the liberation of free [^{3}H]AA (Marshall and McCarte-Roshak, 1992). Lyso PAF:acetyltransferase activity was determined as described previously (Winkler *et al.*, 1993), by measuring the production of [^{3}H]PAF from 1-alkyl-2-lyso-GPC and [^{3}H]acetyl-CoA in broken neutrophils.

PAF production by intact neutrophils. The incorporation of [^{3}H]acetate into 1-radyl-2-lyso-GPC during cell activation was used to quantify PAF biosynthesis (Mueller *et al.*, 1983; Winkler *et al.*, 1993). Cell suspensions (10 × 10^6 cells/ml) in PBS containing 1 mM Ca^{2+} and 1.1 mM Mg^{2+} were incubated at 37° in a volume of 950 μl and exposed to drugs or vehicle for the indicated times. Then, 50 μl of a solution containing [^{3}H]acetic acid (30 μCi) with A23187 (1 or 2 μM final) in PBS with Ca^{2+}, Mg^{2+}, and 1 mg/ml BSA was added to the cell suspensions. After 10 min at 37°, the reactions were terminated by the addition of 1 volume of chloroform/methanol (1:2, v/v), and the lipids were extracted (Winkler *et al.*, 1991). After extraction of total lipids, individual phospholipids were separated by TLC on silica gel G plates developed in chloroform/methanol/glacial acetic acid/water (50:25:8:4, v/v/v/v) and localized by radioscanning (Bioscan). The area corresponding to PAF was then scraped and quantified by liquid scintillation counting. In human neutrophils, ~90% of the 1-radyl-2-[^{3}H]acetyl-GPC produced under these conditions is 1-alkyl-2-acetyl-GPC (Triggiani *et al.*, 1991).

Eicosanoid assays. After stimulation of inflammatory cells, the cells were pelleted by brief centrifugation, and the supernatant fluids were collected. The mole quantities of three different eicosanoids (LTB_4, LTC_4, PGE_2) in the supernatants were determined with enzyme immunoassay kits (Cayman, Ann Arbor, MI) used according to the manufacturer's directions.

Mouse ear inflammation. The inner and outer surfaces of the left ears of BALB/c male mice (six per group) were treated with vehicle or phorbol ester (4 μg/ear, phorbol-12-myristate-13-acetate; Sigma). SB 216754 was applied topically immediately after the challenge, and its effects were compared with those of the dimethylsulfoxide vehicle. After 4 hr, ear thickness was measured as an index of the edematous response, and the myeloperoxidase activity of the inflamed ears was determined on tissue homogenates as an index of cellular infiltration (Griswold *et al.*, 1991).

Protein analysis. Protein concentrations were determined according to the method of Bradford (1976) with reagents purchased from BioRad (Hercules, CA).

Data analysis. Each displayed experiment was performed in triplicate and is representative of two to four experiments performed on different donor animals. The results are mean ± standard error, with statistical analysis performed on original data with Student's *t* test or analysis of variance with Scheffé *post hoc* tests.

Results

Selectivity profiles of SB 212047 and SB 216754. The ability of SB 212047 and SB 216754 to inhibit CoA-IT activity in microsomes of U937 cells in a concentration-dependent manner was determined, using a 10-min preincubation time, as shown in Fig. 1. As initial assessment of time-dependent inhibition, the compounds were incubated with U937 microsomes for 80 min before the activity measurement. As can be observed in Fig. 1, a longer preincubation time decreases the apparent IC_{50} value.

As a test for selectivity, the ability was assessed of these compounds to inhibit other enzymes involved in lipid metabolism. As shown in Table 1, both compounds had minimal effects on several other enzymes, suggesting that their inhibition of CoA-IT activity was reasonably selective. In addition, SB 212047 was found to inhibit the activity of 85-kDa PLA_2, and SB 216754 could inhibit the activities of 5LO and acetyltransferase. However, these effects were observed at higher concentrations than those needed to inhibit CoA-IT and, importantly, were not time dependent (data not shown). These results suggest that only CoA-IT was inhibited by these compounds in a mechanism-based, time-dependent manner.

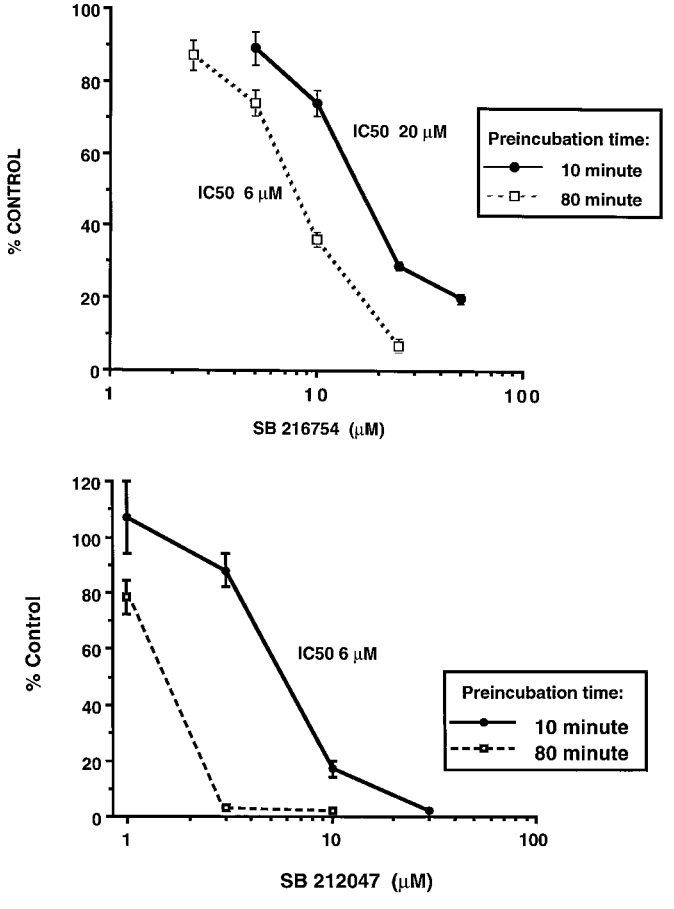


Fig. 1. Inhibition of CoA-IT activity by β-lactams SB 212047 and SB 216754. Microsomes from U937 cells were treated with the indicated concentrations of SB 216754 (A) or SB 212047 (B) for 10 or 80 min and then assayed for CoA-IT activity as described in Experimental Procedures. The control CoA-IT activity was 26 pmol/min/mg. Results are presented as the mean ± standard error of triplicate determinations and are representative of two experiments.

Kinetics of CoA-IT inhibition. Several series of experiments were performed to characterize further the mechanism by which these compounds inhibited CoA-IT activity; these included studies on the kinetics of CoA-IT inhibition, ability of substrate to protect against inhibition, and reversibility of the inhibition.

Microsomes from U937 cells containing CoA-IT activity were treated with different concentrations of β-lactam SB 212047, and the loss of activity was measured over time (Fig. 2A). The data show that the inhibition of CoA-IT was progressive over time and essentially complete by 80 min. In a similar fashion, SB 216754 and two compounds of related structures (SB 216610 and SB 219204; 10-min IC_{50} = 20 and 75 μM, respectively) caused inhibition of CoA-IT activity that was dependent on pretreatment time (Fig. 2B).

To test for the ability of substrate to protect against inhibition of CoA-IT by β-lactam inhibitors, experiments were performed in which 1 μM lyso-PAF substrate was added at the same time as the inhibitor and CoA-IT activity was measured in a 10-min assay. Under such experimental conditions, both SB 212047 (20 μM) and SB 216754 (20 μM) do not inhibit CoA-IT activity (data not shown), suggesting substrate protection is effective.

Experiments were performed to attempt to recover CoA-IT activity after inhibition by β-lactams. Microsomes containing CoA-IT activity were incubated with SB 212047 (40 μM) for 80 min, such that >90% of the activity was inhibited. These microsomes then were washed with PBS and centrifuged (100,000 × *g*, 60 min) three separate times. After each centrifugation, CoA-IT activity was measured and still found to be inhibited by >90% (data not shown). In addition, microsomes of U937 cells containing CoA-IT activity were incubated with β-lactam SB 216754 for 0 or 30 min and then diluted 20-fold directly into assay conditions; CoA-IT activity was measured. As shown in Fig. 3, minimal inhibition of CoA-IT activity is observed at the final concentration of inhibitor if the microsomes are diluted immediately after the addition of the compound. In contrast, when the compound was incubated with the microsomes for 30 min before dilution, inhibition of activity is observed, indicating that inhibition is a time-dependent process. Similar results were obtained with SB 212047 (data not shown).

Finally, the ability of CoA-IT activity to recover from β-lactam inhibition was measured over longer time periods. U937 microsomes were treated with SB 216754 (300 μM) and diluted to decrease the effective concentration of the compound to 15 μM, and then CoA-IT activity was measured over a 6-hr period. The results (Fig. 4) show no indication of recovery of CoA-IT activity over the 6-hr period.

Influence of SB 212047 and SB 216754 on lipid mediator production. We have shown previously that inhibition of CoA-IT activity in inflammatory cells, using competitive inhibitors, resulted in decreases in the ability of those cells to produce inflammatory mediators (Winkler *et al.*, 1995). We

TABLE 1
Effects of SB 212047 and SB 216754 on some enzymes involved in lipid metabolism
CoA-IT IC_{50} was measured after 80 minutes of pretreatment.

Compound	CoA-IT	PLA_2		5LO	CoA-dependent acylation	Acetyltransferase	Cyclooxygenase
		Type II, 14-kDa	Type IV, 85-kDa				
SB 212047	2 μM	NE at 50 μM	10 μM; Not time-dependent	NE 50 μM	NE at 10 μM	Not tested	NE at 50 μM
SB 216754	6 μM	NE at 50 μM	NE at 50 μM	12 μM; Not time-dependent	NE at 10 μM	22 μM; Not time-dependent	NE at 50 μM

NE, no significant effect

extended this study to examine the cellular pharmacology of the irreversible β-lactam compounds. Treatment of human neutrophils with up to 30 μM SB 212047 had no effect on the ability of those cells to produce PAF (20% inhibition compared with A23187 control response; three experiments; p = N.S.). One possible explanation of this lack of cellular effect of this compound is a lack of cellular stability. This hypothesis was tested *in vitro* by the addition of glutathione to mimic the reducing environment within the cell. Glutathione (100 μM) reduced the ability of 30 μM SB 212047 to inhibit CoA-IT in microsomes [CoA-IT activity (mean ± standard error) for control, 16,287 ± 477 dpm; glutathione, 16,153 ± 418 dpm (99%); SB 212047, 677 ± 205 dpm (4%); and glutathione plus SB 212047, 15,466 ± 330 dpm (95%)]. This suggests a possible mechanism by which the compound may lack stability within the cell and thus have no inhibitory effect on PAF production

In contrast to SB 212047, the ability of SB 216754 to inhibit CoA-IT activity was not affected by glutathione treatment, and thus this compound could be predicted to have the potential to block the production of lipid mediators in human inflammatory cells. Fig. 5 shows that SB 216754 inhibited PAF production in neutrophils (IC_{50} = 5 μM). SB 216754 also was able to affect the production of both LTC_4 and PGE_2 in human monocytes, as shown in Fig. 6. Importantly, the effectiveness of SB 216754 for inhibition of the production of these mediators increased with longer pretreatment times (Figs. 5 and 6).

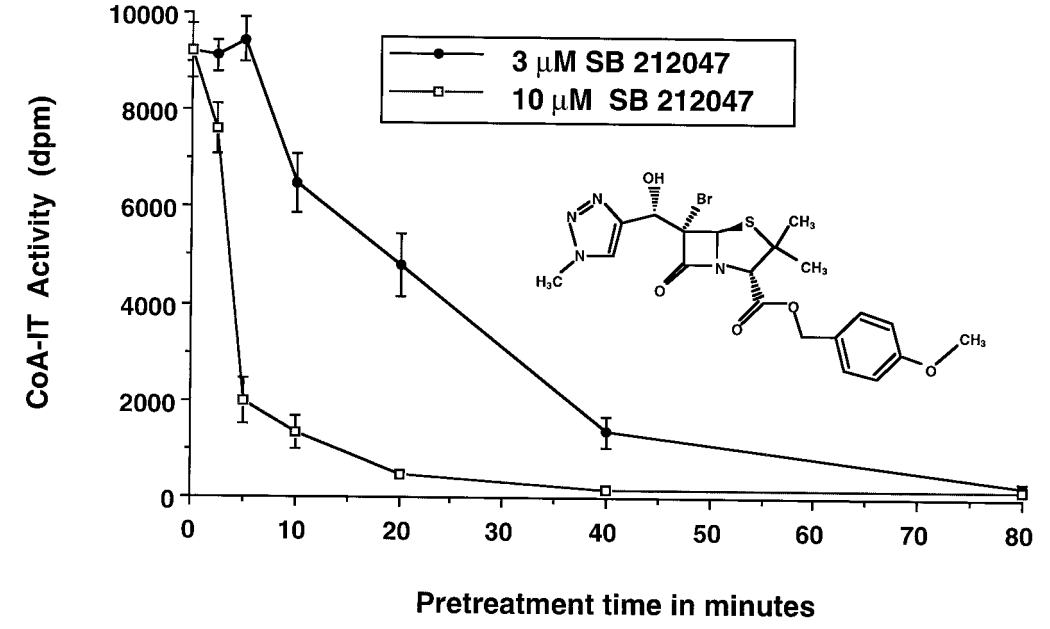
Effect of inhibition of CoA-IT on inflammation *in vivo*. The profile of SB 216754 as an inhibitor of CoA-IT is that of a compound causing a broad reduction in the production of a variety of lipid mediators in isolated inflammatory cells. To test whether this profile would translate into anti-inflammatory effects in an *in vivo* setting, we selected phorbol ester-induced inflammation in the mouse ear, a model of inflammation in which a variety of lipid mediators are produced. In this model, inflammation has been attributed to the production of a variety of lipid inflammatory mediators, including LTs, PGs, and PAF (Griswold *et al.*, 1991; Merlos *et al.*, 1991). We extensively characterized this model, and we and others have shown previously that inflammation can be blocked by inhibitors of PLA_2 and by dexamethasone (Tramposch *et al.*, 1992; Miyake *et al.*, 1993; Marshall *et al.*, 1994, 1995). In addition, we have shown that topical application of the CoA-IT inhibitor SK&F 98625 or SK&F 45905 to the ear prevented both edema and inflammatory cell influx induced by the inflammatory stimulus (Winkler *et al.*, 1995). In a similar fashion, the current study shows that SB 216754 inhibited both indices of the inflammatory response in the mouse ear (Table 2).

Experiments were performed in which animals were treated with SB 216754 and then tissue prepared to examine CoA-IT activity. Because SB 216754 is an irreversible inhibitor of CoA-IT activity, inhibition of activity that occurred *in vivo* would be expected to be detected in a subsequent *ex vivo* assay. In such an experiment, mice were treated with phorbol ester or vehicle and then with SB 216754 (0.75 mg/ear); ear edema and CoA-IT activity were measured after 4 hr. There was a good correspondence between inhibition of inflammatory edema (−73%) and inhibition of CoA-IT activity (−62%) (control, 34 ± 2; phorbol ester, 42 ± 6; phorbol ester plus SB 216754, 37 ± 1 pmol/min/mg). An additional experiment was performed to examine whether this correspondence continued during a chronic inflammatory response. Mice were treated with phorbol ester or vehicle and then with SB 216754 (0.75 mg/ear twice daily for 3 days), after which the tissue content of CoA-IT activity was determined. Phorbol ester treatment increased CoA-IT activity, whereas SB 216754 inhibited this increase by 70% (control, 38 ± 13; phorbol ester, 58 ± 6; phorbol ester plus SB 216754, 45 ± 8 pmol/min/mg). In the same mice, SB 216754 demonstrated statistically significant decreases in both edema (−26%) and myeloperoxidase (−47%). These results strengthen the hypothesis that the anti-inflammatory effects observed with SB 216754 could be ascribed to inhibition of CoA-IT activity.

Discussion

Although much is known about the activity of CoA-IT, key information on its structure and mechanism of action remains to be uncovered. As a working model, we proposed that the mechanism of action of CoA-IT may be similar to that determined for lecithin cholesterol acyl-transferase (Jauhiainen and Dolphin, 1986; Winkler and Chilton, 1993). This proposal is based on inhibitor studies showing that the activity of CoA-IT is inhibited by the serine esterase inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone, the histidine modifier diethyl pyrocarbonate, and agents that modify cysteine residues, such as *N*-ethylmaleimide (Kramer and Deykin, 1983; Winkler *et al.*, 1991). However, this proposed mechanism of action of CoA-IT remains difficult to prove in the absence of purified enzyme with sequence details. One attempt to further our understanding of this enzyme involves the investigation of β-lactams as mechanism-based inhibitors.

A large number of β-lactam-containing compounds were screened for inhibition of CoA-IT, but only a few were found to be inhibitors. The experiments described herein demonstrate that specific β-lactam compounds can inhibit CoA-IT activity with many characteristics used to define mechanism-based inhibition: concentration dependence, time dependence, irreversibility, selectivity, and substrate protection.



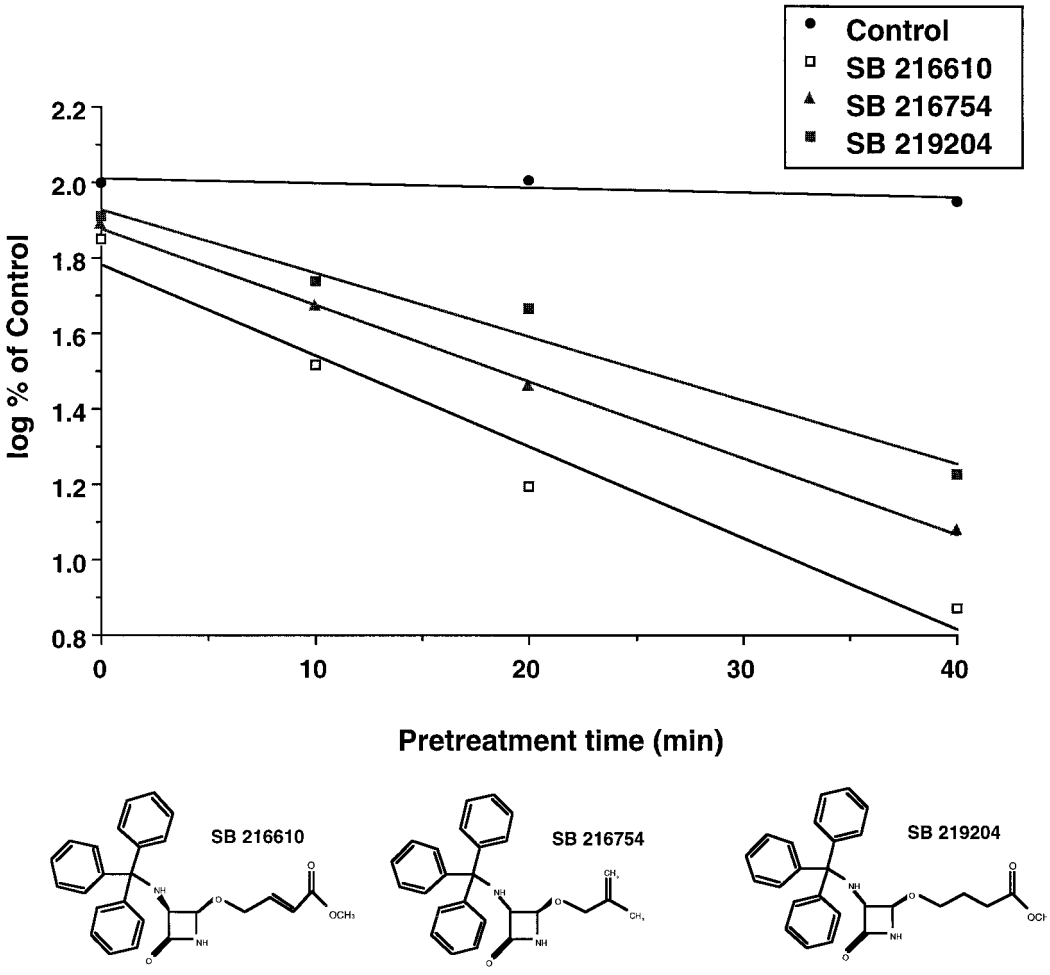


Fig. 2. Time dependence of CoA-IT inhibition. A, Microsomes from U937 cells were treated with 3 or 10 μM SB 212047 for the indicated times and then assayed for CoA-IT activity as described in Experimental Procedures. Results are mean ± standard error of triplicate determinations and are representative of three experiments. The control CoA-IT activity was 24 pmol/min/mg. B, Microsomes from U937 cells were treated with 20 μM SB 216610, 20 μM SB 216754, 80 μM SB 219204, or vehicle for the indicated times and then assayed for CoA-IT activity as described in Experimental Procedures. The control CoA-IT activity was 8464 ± 325 dpm (22 pmol/min/mg). Results are presented as the mean ± standard error of triplicate determinations and are representative of two experiments.

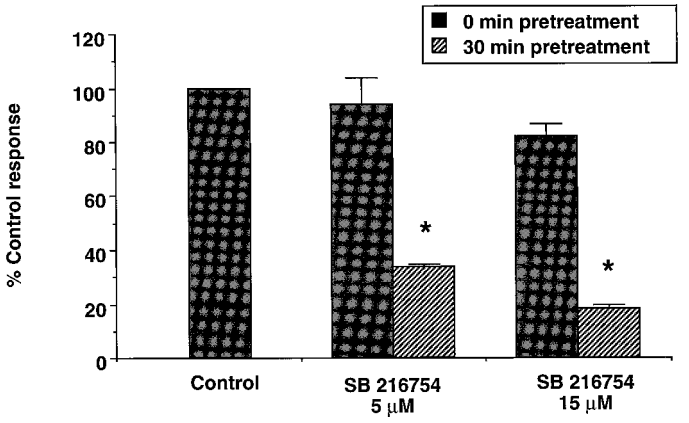


Fig. 3. Effect of dilution on the ability of SB 216754 to inhibit CoA-IT activity. Microsomes from U937 cells were incubated with vehicle, 100 μM SB 216743, or 300 μM SB 216754 for 0 or 30 min, followed by a 20-fold dilution (5 and 15 μM SB 216754 final). CoA-IT activity was determined as described in Experimental Procedures. The control CoA-IT activity was 2526 ± 66 dpm (7 pmol/min/mg). Results are presented as the mean ± standard error of triplicate determinations and are representative of two experiments.

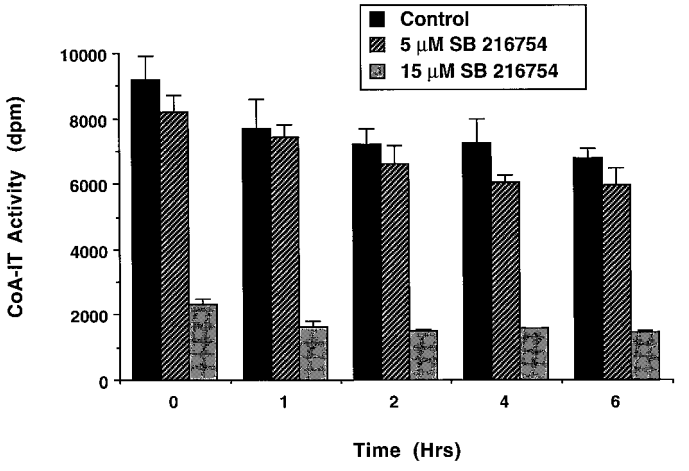


Fig. 4. Lack of recovery of CoA-IT activity after treatment with SB 216754. Microsomes of U937 cells were treated with SB 216754 for 30 min as described in the legend to Fig. 3, diluted, and kept at room temperature for the indicated times before assay for CoA-IT activity. The control CoA-IT activity was 24 pmol/min/mg. Results are presented as the mean ± standard error of triplicate determinations and are representative of two experiments.

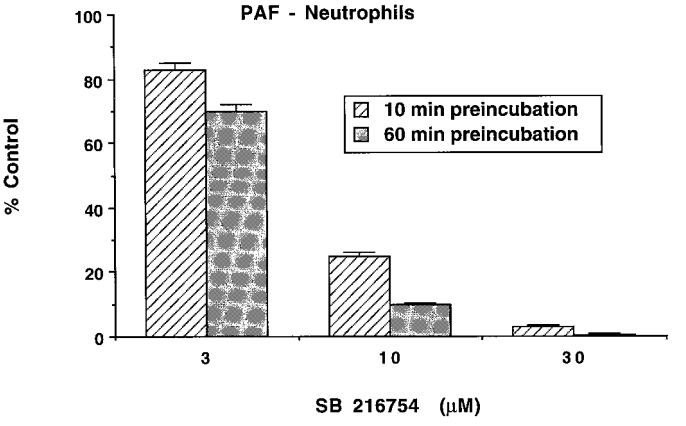


Fig. 5. Effect of SB 216754 on PAF production. Human neutrophils were treated with the indicated concentration of SB 216754 for 10 or 60 min, after which PAF production was measured during stimulation with 2 μM A23187, using [^{3}H]acetic acid as described in Experimental Procedures. Base-line (unstimulated) dpm was 128 ± 12, and the stimulated control was 30,133 ± 153. Results are presented as the mean ± standard error of triplicate determinations and are representative of three experiments.

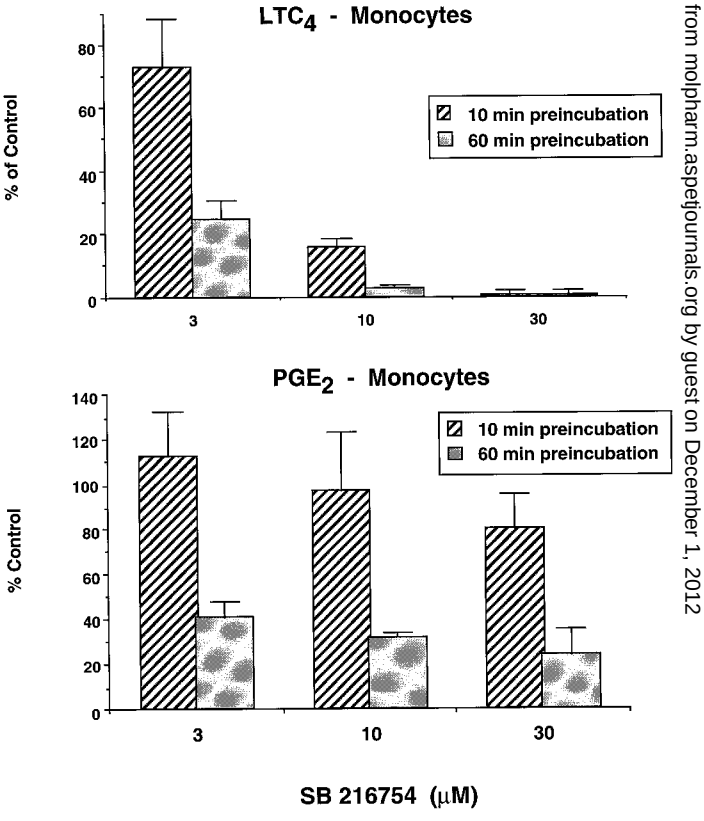


Fig. 6. Effect of SB 216754 on LTC_4 and PGE_2 production. Human monocytes were treated with the indicated concentration of SB 216754 for 10 or 60 min, after which LTC_4 and PGE_2 production was measured during stimulation with 1 μM A23187. Values of LTC_4 and PGE_2 in the stimulated controls were 24.8 ± 7.5 and 5880 ± 1200 pg/ml, respectively. Results are presented as the mean ± standard error of triplicate determinations and are representative of two experiments.

Furthermore, SB 216754 was shown to block the production of inflammatory lipid mediators in human neutrophils and monocytes and to inhibit indices of the inflammatory response *in vivo*. Two major conclusions can be drawn from these results. The first is that these studies provide further support for the critical role that CoA-IT plays in the inflammatory response. The second is that these results show that specific β-lactams can interact with CoA-IT to irreversibly inactivate the enzyme.

Concerning the mechanism by which β-lactams inhibit CoA-IT, we hypothesize the first step to be the opening of the β-lactam by an active site nucleophile, normally involved in the hydrolysis of the *sn*-2 fatty ester of the donor substrate. The resulting acyl-enzyme intermediate then could be a stable, in effect irreversible, complex, or it may undergo rearrangement and further reactions, such as that classically proposed for clavulanic acid (Brenner and Knowles, 1981). The irreversible nature of the complex, if an acyl-intermediate, suggests that water is excluded rigorously from the active site. This would not be surprising for a membrane-bound, lipid-metabolizing enzyme.

TABLE 2

Effects of topically administered compounds on phorbol ester-induced inflammation in mouse ear

Balb/c mice were given phorbol-12-myristate-13-acetate followed by test compound. The edematous response was measured using a thickness gauge 4 hrs after phorbol-12-myristate-13-acetate application. The animals were killed, the inflamed ears were harvested, and myeloperoxidase was extracted and assayed spectrophotometrically as described in Experimental Procedures. Both compounds produced effects significantly different from vehicle treatment.

Compound	Edema at 4 hr ED_{50}	Myeloperoxidase response ED_{50}
	mg/ear	*mg/ear*
CoA-IT SB 216754	0.49	0.48
Steroid dexamethasone	0.06	0.03

Concerning the role of CoA-IT in inflammation, previous studies have shown a link between CoA-IT activity and PAF biosynthesis, both in broken-cell preparations (Uemura *et al.*, 1991; Venable *et al.*, 1991; Winkler *et al.*, 1992) and in whole cells (Sugiura *et al.*, 1990). Moreover, CoA-IT activity seems to be linked to the transfer, and the rate of transfer, of arachidonate between specific subcellular pools (Fonteh and Chilton, 1992; Winkler *et al.*, 1994). Two previously described inhibitors of CoA-IT, SK&F 98625 and SK&F 45905, were able to inhibit arachidonate movement as well as PAF, LT, and PG production (Winkler *et al.*, 1995). These effects in isolated cell systems translated into anti-inflammatory effects in a specific *in vivo* setting of inflammation. The current results with the β-lactam CoA-IT inhibitor SB 216754 support these previous findings. The data showing SB 216754 reduced lipid mediator production in a time-dependent fashion is particularly strong support for the role of CoA-IT in this response.

As with any tool compound, selectivity always is a concern. SB 216754 was found to inhibit 5LO and acetyltransferase at higher concentrations but not in a time-dependent manner. In addition, the effects of this compound on other enzymes, such as those involved in *de novo* phospholipid biosynthesis, are unknown. Thus, cellular and *in vivo* results should be viewed with proper caution. It is encouraging, however, that three structurally diverse compounds that are inhibitors of CoA-IT (SB 216754, SK&F 45905, and SK&F 98625) have the same pharmacological effects in cells and *in vivo*.

In summary, specific β-lactam compounds can inhibit CoA-IT activity in a concentration- and time-dependent manner. Treatment of inflammatory cells with a β-lactam CoA-IT inhibitor resulted in a time-dependent reduction in the ability of those cells to produce inflammatory mediators. The characteristics of inhibition of CoA-IT by the β-lactam compounds support a mechanism of action involving a covalent interaction between the inhibitors and CoA-IT enzyme.

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