Mechanism of Inhibition of LDL Phospholipase A_2 by Monocyclic-β-lactams. Burst Kinetics and the Effect of Stereochemistry

David G. Tew,*[,‡,§] Helen F. Boyd,[‡] Stephen Ashman,[‡] Colin Theobald,[||] and Colin A. Leach[||]

Departments of Molecular Recognition and Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park North, Third Avenue, Harlow, Essex, CM19 5AW, U.K.

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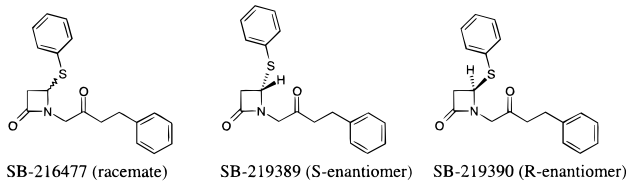
ABSTRACT: Investigation of the inhibition of LDL-associated phospholipase A_2 by monocyclic β-lactams has shown that LDL phospholipase A_2 is capable of hydrolyzing monocyclic-β-lactams by a mechanism which shares many similarities to the hydrolysis of β-lactams by β-lactamases. We believe that this is the first demonstration of a serine-dependent lipase being able to hydrolyze an amide bond. Although 4-(phenylthio)-*N*-(4-phenyl-2-oxobutyl)azetidin-2-one, SB-216477, and its enantiomers are relatively modest covalent inactivators with $k_{obs}/[I] = 46\ M^{-1}\ s^{-1}$ for the *R* enantiomer, analysis of the kinetics of inactivation and reactivation shows that these compounds act as slow-turnover substrates, presumably via an acylation−deacylation mechanism. The detection of a suprastoichiometric burst indicates that the pathway must be branched with the branching giving rise to the slow reactivation via a more stable covalent intermediate. Study of the two enantiomers of SB-216477 shows that LDL-associated phospholipase A_2 is sensitive to the β-lactam stereochemistry at C4. However, a common achiral intermediate is formed along the turnover pathway, and this must be at or immediately prior to the branch point.

LDL phospholipase A_2 (*1*),[1] also known as PAF acetyl hydrolase (*2*), has been shown to be a novel serine phospholipase capable of both degrading PAF and hydrolyzing oxidatively modified phosphatidylcholines (*1*, *3*). The enzyme is found mainly on LDL in plasma. Both of the above enzyme activities have important biological outcomes. PAF is a known inflammatory mediator, and so degradation of PAF would be expected to have an antiinflammatory effect. Oxidation of phosphatidylcholines followed by hydrolysis releases both an oxidized fatty acid and lysophosphatidylcholine (*4*). Lysophosphatidylcholine has been shown to be a chemoattractant for monocytes (*5*) and has been demonstrated to selectively induce the expression of endothelial leukocyte adhesion molecules (*6*). The resulting elevated lysophosphatidylcholine level of oxidized LDL has also been directly related to the ability of oxidized LDL to induce endothelial dysfunction (*7*). Consequently, LDL phospholipase A_2 activity may also act as a proinflammatory enzyme via production of lysophosphatidylcholine. It is of interest, therefore, to derive selective inhibitors of LDL phospholipase A_2 to help differentiate between the possible pro- and antiinflammatory effects of this enzyme and generate antiinflammatory agents acting by a novel mechanism.

Serine lipases share the same catalytic machinery as serine proteases (*8*) in that they have an active site serine residue which, with a histidine and an aspartic or glutamic acid, forms a catalytic triad. The conservation of this catalytic triad suggests that as well as sharing a common mechanism for substrate hydrolysis, that is, formation of a discrete acyl enzyme intermediate via the active site serine hydroxyl group, serine proteases and lipases may well be expected to be inhibited by the same classes of mechanism-based inhibitors. To date, this has been demonstrated for the highly reactive organophosphates and organosulfonates such as diethyl-*p*-nitrophenol phosphate (*9*) and hexadecylsulfonyl fluoride (*10*), fluroketones (*11*), and dichloroisocoumarin (*1*). However, although serine lipases are known to be inhibited by β-lactones (*12*, *13*), inhibition by their amide analogues, β-lactams, has not been observed. We report here the first example of inhibition of a serine lipase by β-lactams. Following observations that LDL-PLA_2 could be inhibited by esters of clavulanic acid (Tew, unpublished observation), we have developed monocyclic-β-lactams as highly specific inhibitors of this enzyme. We have investigated the kinetic and chemical mechanism of inhibition of LDL-PLA_2 by simple monocyclic-β-lactams. The results presented below for SB-216477 and its enantiomers, SB-219389 and SB-219390, suggest a minimal mechanism which shares similarities to that suggested for inhibition of both β-lactamases and elastase by β-lactams.



* To whom correspondence should be addressed.
‡ Department of Molecular Recognition.
§ Current address: SmithKline Beecham Pharmaceuticals, 709 Swedeland Rd., P.O. Box 1539, King of Prussia, PA 19406-0939.
|| Department of Medicinal Chemistry.

[1] Abbreviations: DNPG, 1-decanoyl-2-(4-nitrophenylglutaryl)phosphatidylcholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); LDL, low-density lipoprotein; PAF, platelet activating factor.

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EXPERIMENTAL PROCEDURES

Materials

Ellman's reagent (DTNB) was obtained from Boehringer Mannheim. β-Hydroxybutyrate dehydrogenase and all buffer reagents were obtained from Sigma Chemical Co. Recombinant LDL-PLA_2 was purified by the method of Tew et al. (manuscript in preparation). DNPG was synthesized as published (*14*).

Methods

All curve fitting was achieved using Grafit 3.0 (*15*).

K_i values were determined using a 96 well assay format. Microplates of increasing compound concentration and buffer were made up to a volume of 0.17 mL (50 mM Hepes, 150 mM NaCl, pH 7.4) using a Quatro SP400 robot. Substrate (0.01 mM DNPG, $K_m = 0.01$ mM) and enzyme (100 pM) were added to the plate, and the reaction was monitored at 405 nm and 37 °C for 10 min using a Molecular Devices Tmax plate reader. At the end of the assay, the first 10 points are collected and used in the curve-fitting analysis. Data were fitted to eq 1 with the assumption that the inhibitor is purely competitive:

$$v = V_{max}/(2 + I/K_i) \quad (1)$$

Measurement of the observed rate of inactivation, k_{obs}, for the inactivators was conducted using 96 well microplates. Varying concentrations of inhibitor along with buffer (50 mM Hepes, 150 mM NaCl, pH 7.4) and 0.05 mM substrate were incubated at 37 °C for 5 min prior to the addition of 150 pM enzyme. The reaction was monitored continuously at 37 °C and 405 nm for 40 min using a Molecular Devices Tmax plate reader. k_{obs} was obtained by fitting to eq 2:

$$\text{absorbance} = V_o[1 - \exp(-k_{obs}t)]/k_{obs} + bt + z \quad (2)$$

where V_o is the initial rate, b is the background rate of substrate hydrolysis, and z is a simple adjustment to allow for absorbance not being exactly zero at the start of the experiment. V_o and k_{obs} values were found to have an error of less than 3%.

Enzyme reactivation rates were measured by incubating excess compound (1000-fold) in the presence of 100 nM enzyme for 1 h at 37 °C to allow formation of an enzyme−inhibitor complex. The complex was then diluted 1:1000 into excess substrate (0.1 mM). Enzyme activity was monitored at 400 nm and 37 °C for 1 h using a Hewlett-Packard 8452 spectrophotometer. Reactivation rates were calculated by fitting to eq 3:

$$\text{absorbance} = V_o t + V_f[t + (\exp(-k_{react}t) - 1)/k_{react}] \quad (3)$$

where V_o is the initial velocity, V_f is the final velocity, and k_{react} is the rate of reactivation.

Thiol release was determined by reaction with Ellman's reagent. Quartz cuvettes containing the following were set up: 2 μM purified LDL-PLA_2, 0.1 mM compound, and 0.1 mM Ellman's reagent in buffer (50 mM Hepes, 150 mM NaCl, pH 7.4). In the case of the enzyme−inhibitor reactions, base line data were collected in the presence of enzyme only for 30 min prior to the addition of inhibitor. Following the addition of inhibitor, the reaction was monitored for 3 h at 412 nm and 37 °C using a Hewlett-Packard 8452 spectrophotometer.

β-Keto acid formation was determined by the β-hydroxybutyrate dehydrogenase catalyzed oxidation of NADH. Quartz cuvettes were set up containing the following: 0.1 mM inhibitor, 0.3 mM NADH, 5 units of β-hydroxybutyrate dehydrogenase, 2 μM purified LDL-PLA_2 in buffer (50 mM Hepes, 150 mM NaCl, pH 7.4). The decrease in absorbance was monitored at 37 °C and 340 nm for 1 h using a Hewlett-Packard 8452 spectrophotometer.

Turnover products for analysis by HPLC were prepared by reacting 0.5 mM SB-216477 with 0.02 mM purified enzyme in buffer (50 mM Hepes, 150 mM NaCl, pH 7.4). The reaction was incubated at 37 °C for 15 h before being loaded onto an α Chrom (Upchurch Scientific) C4 wide pore column (2 mm × 15 cm). A linear gradient of 0−100% B over 30 min was used to elute the products (buffer A = water + 0.1%TFA, buffer B = 80% MeCN + 0.1% TFA). Data were acquired with a diode array detector collecting from 200 to 700 nm. HPLC mass spectrometry was run on a Finnigan LCQ system.

To obtain mass spectrometric data on the LDL-PLA_2 covalent intermediate, 5 μM LDL-PLA_2 was incubated with 100 μM SB-216477 for 90 min at 37 °C in 50 mM Hepes, 150 mM NaCl, pH 7.4. Fifty microliters of this reaction was injected onto a 2 × 100 mm C-18 reverse phase column (α Chrom, Upchurch Scientific). Samples were eluted using a 60 min linear gradient (buffer A = water + 0.1%TFA, buffer B = 80% MeCN + 0.1% TFA) at a flow rate of 200 μL min^{-1}. The eluting components were analyzed using a Finnigan LC-Q electrospray mass spectrometer.

4-(Phenylthio)azetidin-2-one was prepared by literature methods as a racemate (*16*), and as the *R* and *S* enantiomers (*17*). 1-Bromo-4-phenylbutan-2-one was prepared as described previously (*18*).

4-(Phenylthio)-N-(4-phenyl-2-oxobutyl)azetidin-2-one, SB-216477. To a cooled (cold water bath) solution of racemic 4-(phenylthio)azetidin-2-one (1.9 g, 11 mmol), tetra-*n*-butylammonium bromide (0.4 g, 1.1 mmol), and 1-bromo-4-phenylbutan-2-one (2.7 g, 11.8 mmol) in dry tetrahydrofuran (75 mL) was added freshly powdered potassium hydroxide (1.8 g, 31 mmol), and the mixture was stirred vigorously for 2 h at ambient temperature. Water was added and the product extracted into ethyl acetate, dried, and evaporated to an oil. Treatment with ether/pet. ether gave the product as a white crystalline solid (1.8 g, 51%): mp 62−64 °C; ^{1}H NMR δ ($CDCl_3$) 2.63 (4H, m, $C\underline{H}_2CO + C\underline{H}_2$-Ph), 2.85 (3H, m, $C\underline{H}_2Ph + \underline{H}_{3a}$), 3.44 (1H, dd, $J = 5.0$, 15.1 Hz, $\underline{H}_{3b}$) 3.73, 4.27 (each 1H, d, $J = 18.5$ Hz, $NC\underline{H}_2$), 5.21 (1H, dd, $J = 2.3$, 4.9 Hz, $\underline{H}_4$), 6.97−7.40 (10H, m, Ph-$\underline{H}$). Found: C, 70.0; H, 5.9; N, 4.4%; $C_{19}H_{19}NO_2S$ requires: C, 70.1; H, 5.9; N, 4.3%.

The enantiomers were prepared similarly, from optically pure 4-(phenylthio)azetidin-2-one. ^{1}H NMR spectra were identical to the racemate:

(S)-4-(Phenylthio)-N-(4-phenyl-2-oxobutyl)azetidin-2-one, SB-219389. Colorless oil, 54% yield. Found: C, 69.9; H, 6.0; N, 4.4%; $C_{19}H_{19}NO_2S$ requires: C, 70.1; H, 5.9; N, 4.3%. $[\alpha]_D = +92.7°$ (*c* 0.49, chloroform, 25 °C)

(R)-4-(Phenylthio)-N-(4-phenyl-2-oxobutyl)azetidin-2-one, SB-219390. Colorless oil, 63% yield. Found: C, 69.3;

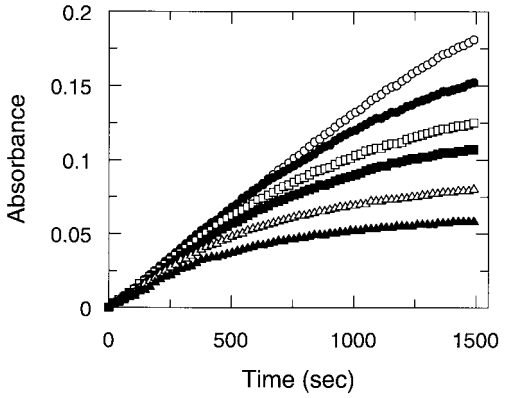


FIGURE 1: Progress curves for the hydrolysis of the LDL-PLA_2 substrate DNGP in the presence of various concentrations of SB-216447: (○) 0 μM, (●) 10 μM, (□) 20 μM, (■) 30 μM, (△) 50 μM, (▲) 70 μM. Every fifth data point is shown for clarity.

H, 5.9; N, 4.4%; $C_{19}H_{19}NO_2S{\cdot}0.2H_2O$ requires: C, 69.4; H, 5.9; N, 4.3%; $[\alpha]_D = -88.7°$ (*c* 0.47, chloroform, 25 °C)

Synthesis of Piperazine Standard. (A) 1-Amino-4-phenyl-2-butanone. A mixture of 1-bromo-4-phenylbutan-2-one (4.54 g, 20 mmol), sodium azide (1.43 g, 22 mmol), dimethylformamide (20 mL), and acetone (140 mL) was stirred at room temperature for 24 h, and then the solvent was removed in vacuo. Aqueous workup and extraction into ethyl acetate gave 1-azido-4-phenyl-2-butanone (3.34 g) as a brown oil.

This material was dissolved in ethanol (80 mL), acidified with a 5% solution of hydrogen chloride in ethanol (25 mL), and hydrogenated over palladium/charcoal at an initial pressure of 50 psi for 3 h. The catalyst was filtered off and the solvent evaporated in vacuo. Trituration of the residue with ether gave 1-amino-4-phenyl-2-butanone hydrochloride (2.49 g) as a white solid.

(B) 2,5-Bis(2-phenylethyl)piperazine. 1-Amino-4-phenyl-2-butanone hydrochloride (199 mg, 1 mmol) and triethylamine (0.15 mL, 1.1 mmol) were dissolved in ethanol (5 mL), and the solution was left to stand at room temperature for 6 days. After evaporation of the solvent, aqueous workup, extraction into dichloromethane, flash chromatography (silica, dichloromethane), and trituration with petroleum ether gave 2,5-bis(2-phenylethyl)piperazine (49 mg) as a white crystalline solid, mp 85−87 °C. Aromatization is presumed to be due to air oxidation; none of the dihydropiperazine was observed: ^{1}H NMR δ ($CDCl_3$) 3.1 (8H, m), 7.1−7.3 (10H, m), 8.28 (2H, s); MS ($APCI^+$) M+H = 289; $C_{20}H_{20}N_2$ requires 288.

RESULTS

Inhibition of LDL-PLA_2. Figure 1 shows the effect of various concentrations of SB-216477, a racemic mixture, on the rate of hydrolysis of a synthetic phospholipid substrate by LDL-PLA_2. It is clear from this figure that SB-216477 is a time-dependent inhibitor of LDL-PLA_2. Figure 2 shows both a plot of the observed rates of inhibition (k_{obs}) by SB-216477 and the initial rates of substrate hydrolysis against concentration. From this, a k_{obs}/[I] value of 25 ± 1.5 M^{-1} s^{-1} can be calculated while the small decrease in the initial rate of substrate hydrolysis suggests that the reversible K_i for Michaelis complex formation prior to the time-dependent step is >100 μM. As SB-216477 is a racemic mixture, it was of interest to see if one enantiomer was more potent

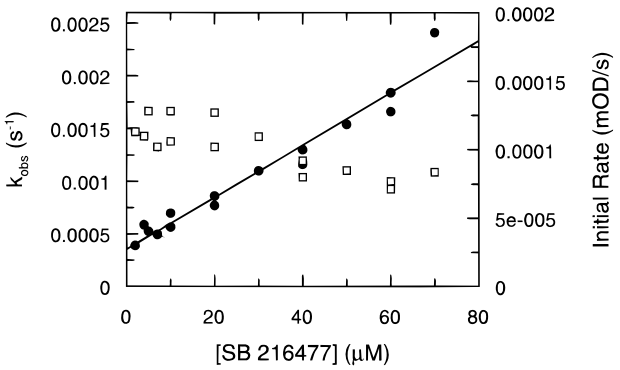


FIGURE 2: Plots of k_{obs} (s^{-1}) (●) and the initial rate of substrate hydrolysis (□) in the presence of varying concentrations of SB-216477. Errors on individual points are no greater than the size of the symbol.

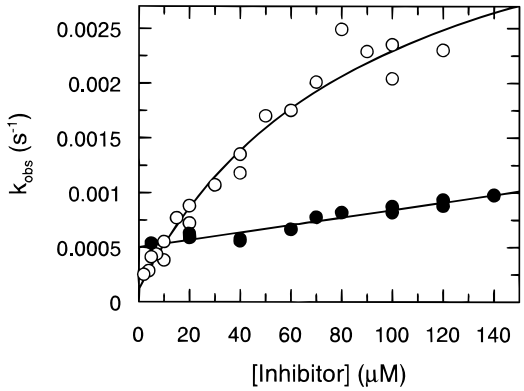


FIGURE 3: Plots of k_{obs} (s^{-1}) against inhibitor concentration for (●) SB-219389 and (○) SB-219390. Errors on individual points are no greater than the size of the symbol.

than the other. Both enantiomers were found to be time-dependent inhibitors of LDL-PLA_2 (data not shown). However, the *R* enantiomer, SB-219390, is significantly more potent than the *S* enantiomer, SB-219389, k_{obs}/[I] = 46 ± 10 and 3.5 ± 0.3 M^{-1} s^{-1}, respectively (Figure 3). It is noteworthy that the plot of k_{obs} vs [SB-219390] shows some curvature, indicating an approach to saturation. This is supported by decreasing initial rates of substrate hydrolysis as [SB-219390] increases which estimate a reversible K_i for SB-219390 of around 60 μM (data not shown).

Enzyme Reactivation. Figure 4 shows the reactivation of LDL-PLA_2 upon dilution of the inhibited enzyme into a large excess of substrate. The two enantiomers, SB-219389 and SB-219390, give rise to slowly reactivating enzyme forms. The reactivation rate for both enantiomers is indistinguishable and equal to 0.0015 ± 0.0003 s^{-1}.

Analysis of LDL-PLA_2-Derived Products from SB-216477. Quantitation of the amount of thiol and NADH-dependent, β-hydroxybutyrate dehydrogenase reactive material derived from reacting LDL-PLA_2 with SB-216477 for 1 h shows that 0.02 mM SB-216477 gives rise to 0.019 mM thiol (by reaction with Ellman's reagent) and 0.024 mM β-oxo acid. It is clear, therefore, from this that 1 equiv of SB-216477 gives rise to 1 equiv of thiol, presumably thiophenol, and approximately 1 equiv of an NADH-dependent, β-hydroxybutyrate dehydrogenase reactive material.

Figure 5 shows the HPLC trace at 215 nm resulting from the turnover of SB-216477 by LDL-PLA_2. SB-216477 has been completely turned over as judged by its absence in the

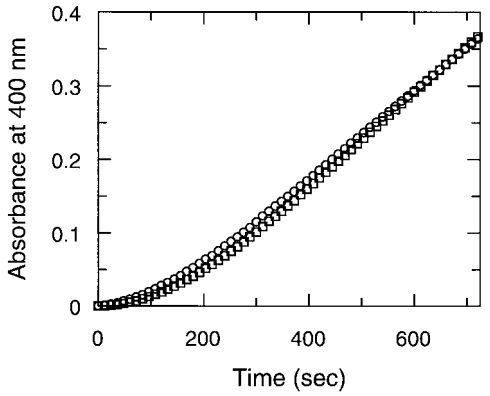


FIGURE 4: Regeneration of enzyme activity by dilution of inactivated LDL-PLA_2 into excess substrate after treatment with either SB-219389 (○) or SB-219390 (□). Enzyme was diluted 1000-fold to achieve 0.1 nM LDL-PLA_2 in 100 μM substrate.

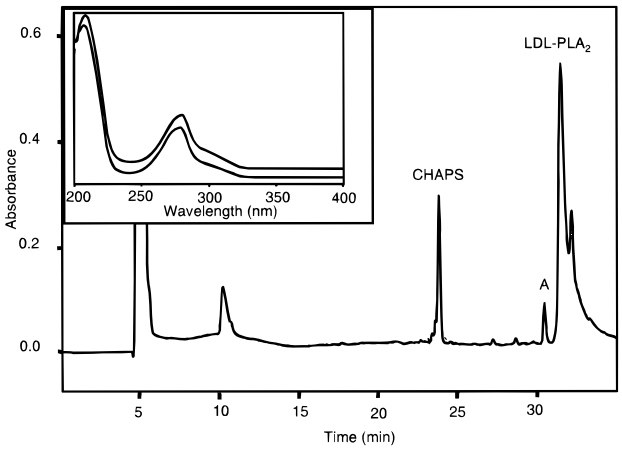


FIGURE 5: Reversed-phase HPLC at 215 nm of the products of turnover of SB-216477 by LDL-PLA_2. The inset shows the UV spectrum of the product peak (peak A) (upper displaced spectrum) and a 2,5-bis(2-phenylethyl)piperazine standard (lower spectrum).

chromatogram (SB-216477 has a retention time of 27 min). Only one new peak is visible. This peak has the same retention time and UV spectrum (see inset) as 2,5-bis(2-phenylethyl)piperazine. In addition, mass spectrometry confirmed that the molecular weight of this compound was 288. No attempt was made to quantitate the amount of 2,5-bis(2-phenylethyl)piperazine formed due to the insolubility of this material at the concentrations required from the turnover experiments performed. It is noteworthy that other products of SB-216477 turnover are not apparent in this HPLC. This is because the 3-oxopropanoic acid formed is expected to appear in the void volume while diphenyl disulfide (formed from oxidative dimerization of the thiophenol released) coelutes with enzyme under the conditions used. The peak at 11 min is not compound related.

Kinetics of Inhibitor Turnover. Figure 6 shows the production of thiol as determined by reaction with Ellman's reagent as a function of time when excess SB-219390 is incubated with varying concentrations of LDL-PLA_2. Not only is thiol released during the incubation as determined above, but the time courses show that an initial burst of thiol release is followed by a slower steady-state phase. The amount of thiol released during the experiment is significantly greater than the enzyme concentration, indicating that multiple turnovers of SB-219390 occur. The burst size and the final steady-state rate are a function of enzyme concentration. Similar data but with a slower approach to steady state are shown for SB-219389 in Figure 7. Back-extrapolation to $t = 0$ from the steady-state phases in Figures 6 and 7 allows the size of the burst to be calculated. The variation of burst size as a function of enzyme concentration is shown in Figure 8. For both SB-219389 and SB-219390, the burst size is a linear function of LDL-PLA_2 concentration, and, from the slope of the plot, 7.2 ± 0.2 mol of thiol is calculated to be released per mole of LDL-PLA_2.

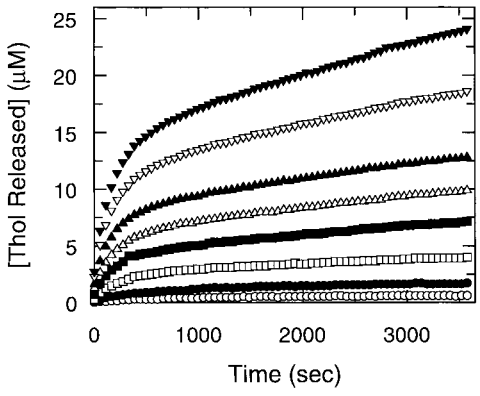


FIGURE 6: Progress curves for the production of thiol from SB-219390 in the presence of 100 μM Ellman's reagent. LDL-PLA_2 concentrations were (○) 0 μM, (●) 0.1 μM, (□) 0.25 μM, (■) 0.5 μM, (△) 0.75 μM, (▲) 1.0 μM, (▽) 1.5 μM, and (▼) 2.0 μM. Every fifth data point is shown for clarity

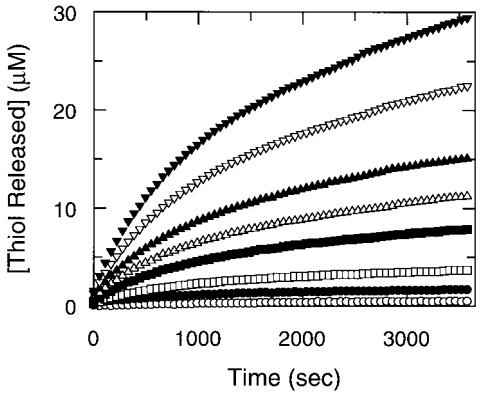


FIGURE 7: Progress curves for the production of thiol from SB-219389 in the presence of 100 μM Ellman's reagent. LDL-PLA_2 concentrations were (○) 0 μM, (●) 0.1 μM, (□) 0.25 μM, (■) 0.5 μM, (△) 0.75 μM, (▲) 1.0 μM, (▽) 1.5 μM, and (▼) 2.0 μM. Every fifth data point is shown for clarity.

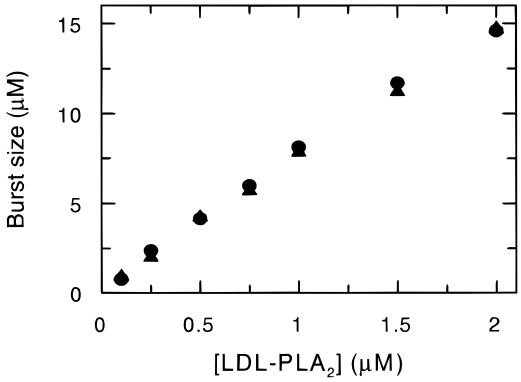


FIGURE 8: Plot of extrapolated burst size vs enzyme concentration for both SB-219389 (▲) and SB-219390 (●). Both data sets fit to a straight line of slope 7.2 ± 0.2 (not shown for clarity).

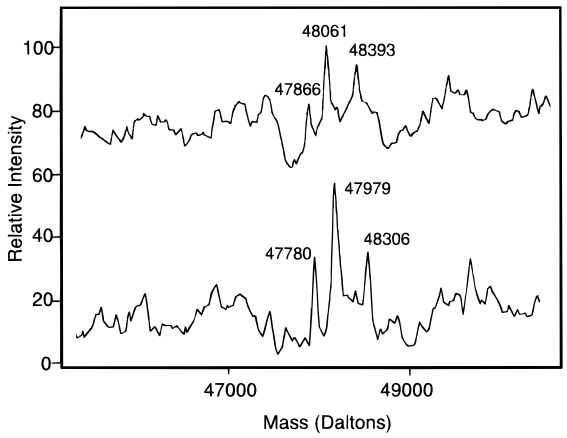


FIGURE 9: HPLC electrospray mass spectometry data for LDL-PLA_2 before (lower trace) and after (upper trace) inactivation with SB-216477. Three peaks are seen due to the heterogeneity of the LDL-PLA_2.

Following β-ketoacid formation using the β-hydroxybutyrate dehydrogenase coupled oxidation of NADH yields similar data to those described above for the production of thiol (data not shown). As following the reaction of Ellman's reagent with thiol yielded higher quality data and was more convenient that using the β-hydroxybutyrate dehydrogenase coupled reaction, detailed analysis of the burst kinetics was performed only using thiol determination.

Mass Spectrometry. The mass spectrum of LDL-PLA_2 obtained by reversed phase HPLC-MS before and after incubation with SB-216477 is shown in Figure 9. The LDL-PLA_2 is clearly heterogeneous, showing three major mass species. Upon incubation with SB-216477, all three LDL-PLA_2 peaks shift by 85 $\pm$ 3 Da. This is consistent with the inactivated LDL-PLA_2 retaining a malonic semialdehyde hydrate at the active site serine after reversed phase HPLC in 0.1% TFA in water. The calculated mass increase for this adduct is 86 Da and is well within the experimentally determined mass increase of 85 $\pm$ 3 Da.

DISCUSSION

The racemic mixture, SB-216477, is a time-dependent inhibitor of the serine-dependent lipase, LDL-PLA_2. The activity of this racemate resides predominantly in the *R* enantiomer, $k_{obs}/[I] = 46 \pm 10\ M^{-1}\ s^{-1}$, with the *S* enantiomer, SB-219389, being more than 10-fold less active. The inhibition kinetics for these enantiomers suggest that the difference in activity is at least in part due to a difference in the initial recognition of the compound by the enzyme as the K_i for the *R* enantiomer is estimated to be 60 μM while that for the *S* enantiomer is >100 μM. The weakness of this interaction precludes better estimation. The time dependency is consistent with an enzyme acylation mechanism as usually seen for the interaction of serine-dependent hydrolases with β-lactams. The rate of enzyme reactivation (putatively hydrolysis of the acyl enzyme complex) of 0.0015 $\pm$ 0.0003 s^{-1} indicates that these molecules are likely to behave as slow-turnover substrates.

Analysis of the products of the LDL-PLA_2-dependent hydrolysis of the racemate, SB-216477, shows that LDL-PLA_2-dependent hydrolysis of SB-216477 yields three products in approximately equimolar concentrations. These products are (1) a thiol, presumably thiophenol as this is the C4 substituent of the β-lactam ring, as determined by reaction with Ellman's reagent; (2) a β-oxo acid, presumably 3-oxopropanoic acid, as determined by the β-hydroxybutyrate dehydrogenase dependent oxidation of NADH; and (3) 1-amino-4-phenyl-2-butanone as determined by HPLC of 2,5-bis(2-phenylethyl)piperazine, the product of dimerization, and subsequent oxidation of this aminoketone. This is indicated in Scheme 1. This minimal mechanism is similar to that proposed for the inhibition of β-lactamases by β-lactam antibiotics (*19*) and serine proteases by β-lactams (*20, 21*). In this mechanism, the β-lactam C4 substituent, in this case thiophenol, is expected to act as a leaving group at some point after (as shown illustrated in Scheme 1), or coincident with, enzyme acylation. Following this, deacylation leads to the loss of the aliphatic imine from the enzyme. This imine is likely to hydrolyze rapidly, giving both 3-oxopropanoic acid and 1-amino-4-phenyl-2-butanone. 1-Amino-4-phenyl-2-butanone can then cyclize to give 1,4-dihydro-2,5-bis(2-phenylethyl)piperazine which is oxidized slowly by air to give the aromatic 2,5-bis(2-phenylethyl)-piperazine as indicated.

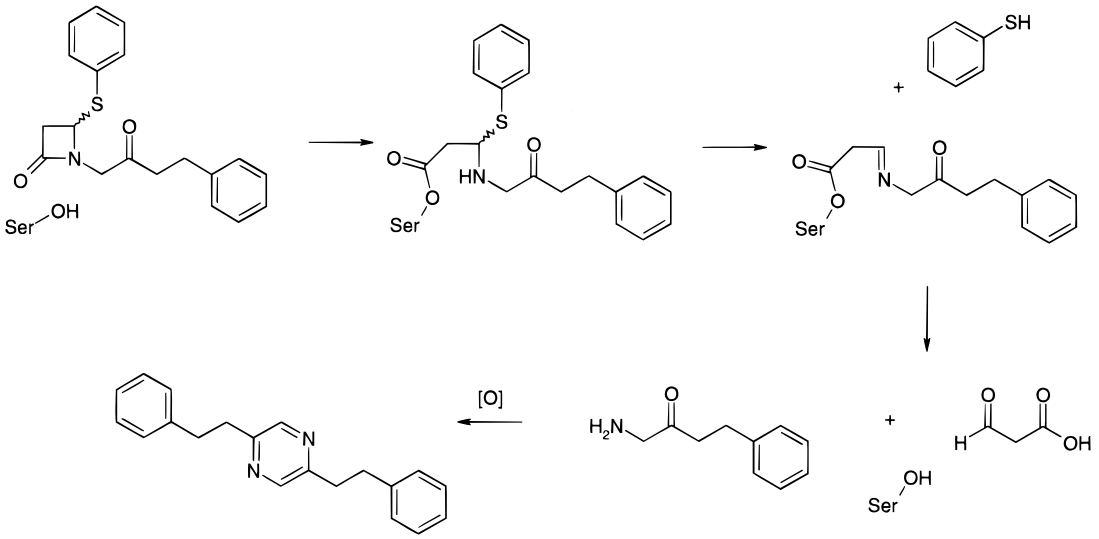
The chiral center at C4 of the β-lactam ring in the pair of enantiomers, SB-219389 and SB-219390, offers us a potential probe of this proposed mechanism. While the C4 substituent is in place, it is possible that the retained chirality at this position will be reflected in a difference in the kinetics observed for the two enantiomers. Once the C4 substituent is lost, any intermediates at, or after, this point in the mechanism will be identical for both enantiomers and so should be kinetically indistinguishable.

Enzyme reactivation occurs sufficiently slowly to be measured by a dilution experiment (Figure 4). Both enantiomers give rise to an inactivated enzyme form which reactivates with the same rate constant, 0.0015 $\pm$ 0.0003 s^{-1}. The equality of this rate constant suggests that the slow step in reactivation occurs after loss of thiophenol and is consistent with reactivation from a common and hence achiral enzyme-bound intermediate.

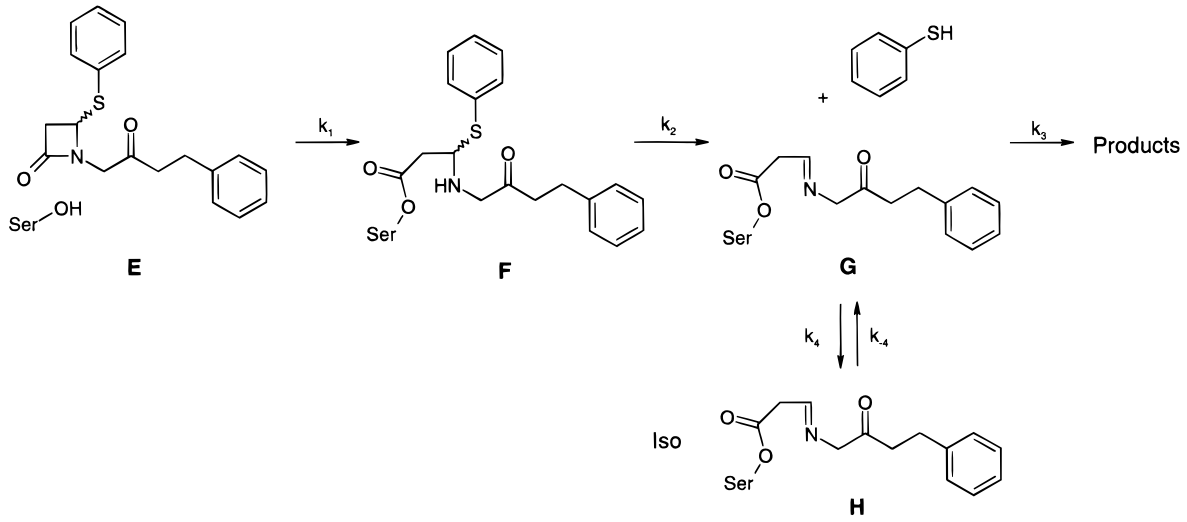
As discussed above, the reactivation rate constant is in keeping with the fact that both enantiomers behave as slow-turnover substrates of LDL-PLA_2. The time courses for both SB-219389 and SB-219390 show marked deviation from linearity with a large burst of thiol release being followed by a much slower steady-state phase. The rate of turnover of both enantiomers during the steady-state phase is in accordance with the rate of enzyme reactivation, suggesting that the rate-determining step during the steady-state phase is regeneration of free enzyme.

The hydrolysis of β-lactams by serine-dependent hydrolases is likely to occur via a Ping-Pong mechanism with the acyl enzyme intermediate being the modified enzyme form required by this mechanism. If the rate of formation of the acyl enzyme intermediate is significantly faster than the rate of hydrolysis to regenerate free enzyme, then burst kinetics will be observed for the product released upon enzyme acylation. This is simply an analogy of the reaction of chymotrypsin with *p*-nitrophenylacetate (*22*). However, for the simple case of the pre-steady-state burst observed in some Ping-Pong reactions, the size of this burst is constrained to be equal to the enzyme concentration. This is clearly not the case for either enantiomer where the observed burst of thiol is approximately 7.2-fold greater than the enzyme concentration. The observation of a pre-steady-state burst

Scheme 1



Scheme 2

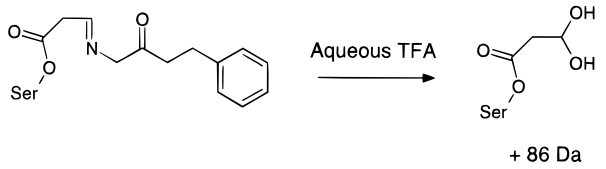


which is much larger than the enzyme concentration has been ascribed to systems where a branched pathway for substrate turnover is involved (*23*−*25*). Thus, this result suggests that the hydrolysis of SB-219389 and SB-219390 by LDL-PLA_2 occurs via such a branched pathway.

The chirality of SB-219389 and 219390 offers us a probe of the branch point in the pathway for turnover of this pair of enantiomers. The fact that the rate of deacylation of LDL-PLA_2 is essentially identical following acylation by either enantiomer suggests that deacylation occurs from a common achiral enzyme intermediate. If we consider the general mechanism described by Waley (*24*), then it is conceivable that the branch point occurs immediately prior to deacylation as shown in Scheme 2. Branching at enzyme form E, i.e., isomerization of free enzyme (*25*), is ruled out by the increase in burst rate with increasing concentration of either enantiomer (data not shown). Similar burst kinetics are seen when the formation of β-keto acid is followed using the β-hydroxybutyrate dehydrogenase coupled oxidation of NADH. That a similar burst is seen under these conditions shows that the deceleration of product formation is not simply due to nonspecific activation of LDL-PLA_2 by malonic semialdehyde or product inhibition as under these conditions the product aldehyde is removed rapidly.

The criteria for the observation of a greater than stoichiometric burst have been discussed in detail by Waley (*24*). In general terms, the equilibrium representing the branch to the dead end complex must be established on a time scale which is slow in comparison to the rate constants for the steps in the unbranched part of the pathway. The rate of reactivation is equal to k_{-4}. We have already established that k_{-4} is indistinguishable for both SB-219389 and SB-219390; thus, enzyme forms G and H should correspond to modification by an achiral species and are downstream of the loss of thiophenol. The difference between enzyme forms G and H is not entirely clear from this work. If G has the structure shown, then there are at least three possibilities for H. Two of these possibilities involve isomerization of the imine double bond, either to lie between C3 and C4 of what was the β-lactam ring or to give the isomeric imine in conjugation with the keto group of what was the β-lactam N1 substituent. The third possibility is an enzyme conformational change which protects H from hydrolysis. Isomerization of the double bond to C3/C4 can be ruled out. The proposed aminoacrylate intermediate would be expected to have a significant absorbance beyond

Scheme 3



280 nm as seen for inactivated β-lactamases (*26*). The UV spectrum of inactivated LDL-PLA_2 shows no appreciable change relative to active LDL-PLA_2, and so it is highly unlikely that an aminoacrylate intermediate is formed. UV data do not help distinguish between a conjugated imine intermediate or a conformational change. The conformational change is perhaps favored, however, as it is not immediately obvious why isomerization of the imine would stabilize the proposed acyl enzyme adduct. Attempts to further define the structure of H by direct electrospray mass spectrometry have been unsuccessful. Reversed phase HPLC mass spectrometry shows that after HPLC in aqueous TFA the enzyme-bound intermediate has a mass of 85 $\pm$ 3 Da. This is consistent with modification of the enzyme by a malonic semialdehyde hydrate fragment derived from the β-lactam ring. Although this would be consistent with enzyme forms G and H being simply malonic semialdehyde hydrate esters, it is also consistent with the proposed imine structures in Scheme 2 being hydrolyzed under the acidic denaturing conditions used for the HPLC as shown in Scheme 3. The work presented here is unable to discriminate between these two possibilities. However, results on related compounds (Tew et al., manuscript in preparation) show that the nature of the β-lactam N substituent markedly alters the reactivation rate of LDL-PLA_2 after inactivation, thus indicating that the β-lactam N substituent must be present in the inactivated enzyme complex. Consequently, we believe that enzyme forms G and H are reasonable structures for the intermediates described in this work.

Clearly, enzyme form E, which is a noncovalent complex, experiences the chirality at C4. The existence of this complex is supported by the approach to saturation seen for SB-219390. Enzyme form F also experiences the chirality at C4. However, there is no direct evidence for the formation of this covalent intermediate in this work. It is conceivable that loss of thiophenol occurs in a concerted fashion along with β-lactam ring opening. However, others (*27*) have argued, based upon stereoelectronic grounds, that concerted loss of C4 substituents upon β-lactam hydrolysis is only possible for one stereochemistry at C4. Thus, at least one enantiomer must result in a discrete acyl enzyme complex being present with the C4 substituent in place. It is conceivable that this mechanistic difference is manifested as the difference seen in the behavior of the two enantiomers of SB-216477. However, it is likely that enzyme form F must occur for at least one, if not both, enantiomers.

The monocyclic β-lactams discussed in this work represent the first examples of inhibition of serine-dependent esterases by β-lactams. These particular compounds, while being only modest inhibitors, show striking similarities in their mechanism of action to that seen for the inhibition of β-lactamases by β-lactams in that they are inhibitory by virtue of being slowly hydrolyzed substrates involving a branched pathway to a more stable covalent enzyme intermediate. As inhibitors of LDL-PLA_2, these compounds should provide a platform for the design and synthesis of more potent inhibitors to probe the role of this enzyme in inflammation and PAF degradation.

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