Efficient Inhibition of Class A and Class D β-Lactamases by Michaelis Complexes*

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A 6-alkylidiene penam sulfone, SA-1-204, is an efficient inhibitor of both SHV-1 and OXA-1 β-lactamases with \( K_f = 42 \pm 4 \text{ nM} \) and \( 1.0 \pm 0.1 \text{ μM} \), respectively. To gain insight into the reaction chemistry of SA-1-204, the reactions between this inhibitor and SHV-1 and OXA-1 were studied by Raman spectroscopy in single crystals and in solution. Raman signatures characteristic of the unreacted β-lactam ring show that in both phases the inhibitor binds as a noncovalent Michaelis-like complex. This complex is present as the major population for periods of up to an hour. On longer time scales, the Raman data show that β-lactam ring opening eventually leads to a complex mixture of reaction products. However, the data clearly demonstrate that the key species for inhibition on the time scale of bacterial half-lives is the noncovalent complex preceding acylation.

The clinical and commercial importance of β-lactamase inhibitors has led to extensive mechanistic studies and structure/activity correlations (1, 2). All clinically important reactions of β-lactamase inhibitors, such as tazobactam, sulbactam, and clavulanic acid, involve β-lactam ring cleavage during acylation of an active-site serine. Other drugs being investigated, such as the boronic acid and phosphonate inhibitors, mimic either the acylation or deacylation transition state of β-lactam ring cleavage. Accordingly, these inhibitors result in a covalently bonded transition state that involves phosphorylation (3) or boronation (4, 5) of the active-site serine residue of class A, C, and D β-lactamases. Only a few novel β-lactamase inhibitors that are not based on a β-lactam core structure and do not chemically react with the active-site serine have been reported (5–7). However, such competitive inhibitors usually have been designed for class C enzymes, and further studies are needed to improve the biological activity beyond that achieved by mechanism and transition state inhibitors.

Using two forms of Raman spectroscopy, we demonstrate that a 6-alkylidiene penam sulfone (Fig. 1) inhibits SHV-1 and OXA-1 without covalent modification of the active-site serine or cleavage of the four-member β-lactam ring. The mode of inhibition occurs on time scales that are relevant for bacterial replication. Such an observation is unprecedented in that, thus far, β-lactam inhibitors and antibiotics have been designed to irreversibly react with β-lactamase enzymes so they cannot proceed to cleave the β-lactam ring of penicillins.

**MATERIALS AND METHODS**

**Inhibitors**—SA-1-204 was synthesized as previously summarized (8). A comprehensive synthesis is provided in the supplemental material. For Raman crystallography, a stock solution of the inhibitor at 20 mM in 2 mM HEPES buffer (pH 7.0) was prepared for “soak in” experiments with the protein crystals. For Raman solution studies, a stock solution of the inhibitor at 2 mM in 2 mM HEPES buffer (pH 7.0) was prepared. Antimicrobial susceptibility testing and determination of kinetic parameters were performed according to Helfand et al. (9). Briefly, kinetic constants were measured by continuous assays at room temperature in 20 mM phosphate buffer (pH 7.4) using an Agilent 8453 diode array spectrophotometer. \( K_I \) values were determined by competing increasing concentrations of SA-1-204 against the colorimetric substrate nitrocefin at 4–6 times the Michaelis constant (\( K_m \)) for the enzyme. Enzyme concentrations were maintained at 0.2 μg/ml, and three experimental determinations were made for each unique nitrocefin/inhibitor concentration. Substrate-velocity curves in the presence of varying concentrations of inhibitor were analyzed by nonlinear regression using a competitive model of inhibition (for an example see supplemental Fig. 1). For both SHV-1 and OXA-1, fitting the progress curves to a noncompetitive inhibition model resulted in an undefined \( K_I \).

**Protein Isolation, Purification, and Crystallization**—The SHV-1 and OXA-1 β-lactamases were isolated by periplasmic fractionation and purified by preparative isoelectric focusing as described previously (3, 10, 11). An additional gel filtration HPLC (high pressure liquid chromatography) purification step was performed using a Sephadex Hi Load 26/60 column (GE Healthcare) and elution with 50 mM sodium phosphate buffer (pH 7.4). Following purification, the respective β-lactamases were crystallized per the protocol of Kuzin et al. for SHV-1 (10) or Sun et al. for OXA-1 (11). The resulting crystals were typically 300 × 300 × 300 μm in size for SHV-1 or 500 × 200 × 200 μm in size for OXA-1.

**Raman Crystallography**—The Raman microscope system has been described previously (12, 13). Briefly, protein crystals were transferred from the mother liquor solution into a 4-μl hanging drop (0.1 M HEPES, pH 7.0). Spectra of the apo-β-lactamase protein crystals were obtained, and subsequently we...
infused the inhibitor into the protein crystal by adding 1 μl of the inhibitor solution to the drop to achieve a final drop volume of 4 μl and a final inhibitor concentration of 5 mM. Spectra of the β-lactamase-inhibitor complex were then acquired serially every 2–3 min following addition of the inhibitor.

For solution studies, 3 μl of a 500 μM solution of the β-lactamase in 2 mM HEPES (pH 7.0) was placed on a siliconized quartz coverslip and mounted in the hanging drop setup. Using the 20× objective of the Raman microscope, spectra of the apo-β-lactamase were obtained, and subsequently 1 μl of the inhibitor was added to achieve a final drop volume of 4 μl and a final inhibitor concentration of 500 μM. For the apo-β-lactamases and enzyme/inhibitor complexes, five spectra were averaged with an acquisition of 60 s for each. The concentration of the inhibitor was checked using a Raman intensity/concentration calibration curve.

Calculations—Ab initio quantum mechanical calculations were performed to predict the Raman spectra of SA-1-204 and model intermediate compounds using Gaussian 03 (14). Calculations were performed at the DFT5 level using the 6-31+G(d) basis set. DFT calculations were performed with Becke’s three-parameter hybrid method using the correlation functional of Lee, Yang, and Parr (B3LYP) (with 20% Hartree-Fock exact exchange mixing).

RESULTS AND DISCUSSION

Inhibitory Activity of SA-1-204—SA-1-204 demonstrated nanomolar affinity for SHV-1 β-lactamase with $K_I = 42 ± 4$ nM and micromolar affinity for OXA-1 with $K_I = 1.0 ± 0.1$ μM. As such, this inhibitor was 8-fold more potent than tazobactam ($K_I = 330 ± 20$ nM) in inhibiting SHV-1 and 400 times more potent than tazobactam ($K_I = 380 ± 27$ μM) in inhibiting OXA-1. This finding is in good agreement with previous inhibition studies that found SA-1-204 to have high activity against class C P99 and class A TEM-1 with $IC_{50} = 1$ and 40 nM, respectively (8). Minimum inhibitory concentrations using SA-1-204 at 4 μg/ml with increasing concentrations of piperacillin as the partner β-lactam showed that this combination was more potent than increasing concentrations of piperacillin and 4 μg/ml tazobactam against Escherichia coli bearing blaSHV-1 (1024/4 versus 64/6 μg/ml). In contrast, no difference was seen when comparing piperacillin/SA-1-204 and piperacillin/tazobactam against E. coli DH10B expressing blaOXA-1 (512/4 μg/ml).

Raman Spectra of the Inhibitor SA-1-204—The spectrum of the free ligand SA-1-204 shown in Fig. 2 is dominated by a feature at 1696 cm$^{-1}$ because of a methylene stretch from the substituent at the 6′-position of the β-lactam. Ab initio quantum mechanical calculations (supplemental Fig. 2) and comparisons to the model compounds 2-phenylindolizine and 2-(2-thienyl)indolizine6 have assigned the “sharp” modes at 1590 and 1570 cm$^{-1}$ to the methylene stretch coupled to pyridine modes, 1216 cm$^{-1}$ to a pyridine ring mode, and 998 cm$^{-1}$ to a phenyl ring mode. The characteristic feature at 1781 cm$^{-1}$ that corresponds to the C=O group of the intact lactam ring is particularly important. According to previous reports of reactions between β-lactamases and inhibitors tazobactam, sulbactam, and clavulanic acid, the absence of this peak confirms opening of the lactam ring and acylation of the enzyme (15, 16).

Spectra for the β-Lactamase Complexes—One inherent complication of soaking inhibitors into protein crystals is that the ligand may not have optimal access to the enzyme active site. Previously, Helfand et al. (15) demonstrated that meropenem rapidly diffuses into β-lactamase crystals to form an acyl enzyme species. Meropenem and the inhibitor used in this study, SA-1-204, possess similar molecular weights and substitution patterns at C-6′ and C-3; consequently, access to the β-lactamase active site should be similar if not identical.

Fig. 3 shows the difference spectra for the inhibitor SA-1-204 as it soaks into the SHV-1 β-lactamase crystals at 10 min (top) and 40 min (bottom). The intense ligand modes remain intact. The methylenic double bond stretch at 1696 cm$^{-1}$, a highly characteristic feature, is expected to change markedly when the β-lactam ring opens and the hybridization at C-6′ changes. This is supported by the observation that the compound 3-(2-pyridyl)-acrylic acid, which models the species formed by open-

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5 The abbreviation used is: DFT, density functional theory.

6 M. Kalp and P. R. Carey, unpublished data.
ing of the β-lactam ring, has its intense double bond stretching frequencies near 1620 and 1645 cm\(^{-1}\) (supplemental Fig. 3). Nonetheless, both the 1696 cm\(^{-1}\) feature and the characteristic lactam carbonyl at 1772 cm\(^{-1}\) remain intact. Taken together, these data show the presence of a large population of unreacted inhibitor in the crystal. In addition, there is evidence for a minor population of acyl enzymes (compare Figs. 2 and 3 in the 1600 and 1300 cm\(^{-1}\) regions) that likely represents a mixture of intermediates on the reaction pathway.

Similar Raman crystallographic experiments were performed using crystals of the class D oxacillinase, OXA-1. Although SA-1-204 inhibits OXA-1 in micromolar concentrations, we wondered whether inhibition was due to simply “blocking” the active site as in class A SHV-1 or readily acylating the active-site serine 67. The spectrum of the ligand reacting in a crystal of OXA-1 is shown at 10 min (top) and 40 min (bottom) in Fig. 4. The results are similar to those obtained for the reaction between SHV-1 and SA-1-204, and again, there is no indication that the lactam ring has opened. This is remarkable considering the dissimilarity between the active sites of the class A and D β-lactamases. Class D β-lactamases share less than <20% amino acid identity with the more prevalent and better understood class A enzymes and differ mechanistically by use of a carboxylated lysine side chain for catalysis (1). In particular, Sun et al. (11) have commented on the lack of recognition elements in the OXA-1 β-lactam binding site, such as those corresponding to residues 104, 132, and 244 in class A that are involved in recognizing and binding β-lactam substrates.

Raman Solution Difference Spectra for β-Lactamase Intermediates—In proposing such a controversial mechanism of inhibition, it is essential to compare the results obtained in crystals with experiments undertaken in solution. This removes any doubt that the results in single crystals may sometimes contain artifacts because of, for example, the effects of close packing within the crystals. Fig. 5 compares the Raman difference spectra of free ligand SA-1-204 in 0.1 M HEPES (pH 7.0) with that of the ligand bound to SHV-1 β-lactamase. In the latter, the enzyme and ligand are present at 500 μM in a 4-μl hanging drop. Using the Raman microscope, the spectrum of the bound SA-1-204 was followed for 30 min using an acquisition time of 60 s × 5 at each time point.

The data obtained from the solution studies shown in Fig. 5 agree with those for SA-1-204 bound to a crystal of SHV-1 (Fig. 3) in that the majority of bound ligand molecules are unreacted, as shown by the characteristic β-lactam carbonyl near 1772 cm\(^{-1}\) and the intense C=C symmetric stretch at 1695 cm\(^{-1}\). Similarly, there appears to be a slow chemical reaction, because minor new peaks grow in with time, such as the feature near 1450 cm\(^{-1}\). In the first data set, from 0 to 5 min, these peaks are barely detectable. The ability to obtain high quality and repro-
Michaelis Complex Inhibits \( \beta \)-Lactamase

Michaelis-like complexes in crystals of M69 variants of SHV-1 (private communication). Structural similarities with the inhibitor used in this study suggest that these compounds may also be slow to acylate the \( \beta \)-lactamase. Thus, the need to obtain crystallographic evidence at earlier time points is essential to our understanding of how the Michaelis complex can result in such potent inhibition. Exploring this unique mechanism is critical to the further development of a universal \( \beta \)-lactamase inhibitor.

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Slowly Formed Species—Solution samples were stable for only 30 min, precluding studies at long reaction times. However, we were able to collect crystal data for 4 and 8 h soaks. The resultant difference spectra are highly detailed and are different from those seen in Figs. 3 and 4 (data not shown). It is possible that we detected the bicyclic aromatic species (see reaction Scheme 1 in the supplemental material) formed by the attack of the pyridine nitrogen on the iminium species, as reported in the x-ray studies by Knox et al. (17); these authors soaked a class C enzyme with 9 mM inhibitor for 3.5 h. We attempted to generate a model for the complex by methanolysis of the 6-alkylidiene penem sulfoxone SA-1-204; however, the Raman spectrum from the methanolysis product did not resemble that for the crystal product after the 4- or 8-h soaks. Thus, the details of the chemistry involved over longer time periods are not understood. However, the crucial point is that complex product formation only occurs in significant amounts in the time scale of hours in solution as in crystals. On the scale of bacterial half-life of 20–30 min, the great majority of active sites must be blocked by noncovalent, unreacted complexes in which this \( \beta \)-lactam ring is intact. Interestingly, crystals of SHV-1 S70G, in which the active-site serine is replaced by a glycine, showed no evidence for the formation of complex reaction products during a 1-h soak (data not shown).

Impact on Future Studies—The results of this study further stress the need to validate the inactivation mechanism between novel \( \beta \)-lactamase inhibitors and \( \beta \)-lactamases with the highly synergistic approach of combined Raman and x-ray crystallography. In all known reports of the reactions between \( \beta \)-lactam inhibitors and \( \beta \)-lactamases, the active-site serine is readily acylated by the inhibitor. For the first time, we have reported an efficient inhibitor of class A and D enzymes that does not achieve inhibition by acylating the enzyme but by blocking the active site as a noncovalent Michaelis complex. This finding is crucial for the rational design of future generation 6-alkylidiene penam sulfoxones and 7-alkylidenecephalosporins, as key binding interactions responsible for the Michaelis complex are different from the interactions responsible for stabilizing an acyl enzyme species.

Other 6-methylidene penems bearing 6,5-bicyclic heterocycles were recently reported as broad spectrum \( \beta \)-lactamase inhibitors (18, 19), and for these, Dr. M. Helfand has observed...