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Why the Extended-Spectrum β -Lactamases (ESBLs) SHV-2 and SHV-5 are “Hyper-Susceptible” to Mechanism-Based Inhibitors

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Abstract

Extended-spectrum β -lactamases (ESBLs) are derivatives of enzymes such as SHV-1 and TEM-1 that have undergone site-specific mutations that enable them to hydrolyze, and thus inactivate, oxyimino-cephalosporins, such as cefotaxime and ceftazidime. X-ray crystallographic data provide an explanation for this in that the mutations bring about an expansion of the binding pocket by moving a β -strand that forms part of the active site wall. Another characteristic of ESBLs that has remained enigmatic is that they are “hyper-susceptible” to inhibition by the mechanism-based inactivators tazobactam, sulbactam and clavulanic acid. Here, we provide a rationale for this “hyper-susceptibility” based on a comparative analysis of the intermediates formed by these compounds with wild-type (WT) SHV-1 β -lactamase and its ESBL variants SHV-2 and SHV-5, which carry the G238S and G238S/E240K substitutions, respectively. A Raman spectroscopic analysis of the reactions in single crystals shows that, compared to WT, the SHV-2 and SHV-5 variants have relatively higher populations of the stable *trans*-enamine intermediate over the less stable and more easily hydrolysable *cis*-enamine and imine co-intermediates. In solution, SHV-2 and SHV-5 also form higher populations of an enamine species compared to SHV-1 as detected by stopped-flow kinetic experiments under single turnover conditions. Moreover, a simple Raman band shape analysis predicts that the *trans*-enamine intermediates themselves in SHV-2 and SHV-5 are held in more stable, rigid conformations compared to their *trans*-enamine analogs in WT SHV-1. As a result of this stabilization, more of the *trans*-enamine intermediate is formed, which subsequently lowers the K_I values of the mechanism-based inhibitors up to 50-fold in SHV-2 and SHV-5.

Extended-spectrum β -lactamase (ESBL)-producing gram-negative organisms are an increasing cause of healthcare-associated infections. *Escherichia coli*, *Klebsiella*

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Supporting Information

Steady-state kinetic parameters for SHV-1, SHV-2, and SHV-5 with ampicillin and nitrocefin and SHV-2 and SHV-5 with cefotaxime and ceftazidime; difference spectra of SHV-5 with tazobactam, sulbactam, and clavulanic acid. This material is available free of charge via the Internet at <http://pubs.acs.org>.

pneumoniae, and *Proteus* spp. are the most common ESBL-producing pathogens recognized in the United States (1). The widespread use of oxyimino-cephalosporins, which were introduced in the 1980s to treat antibiotic-resistant bacteria, is believed to be a major contributor to the emergence of ESBL-producing organisms. ESBL-producing organisms are able to hydrolyze oxyimino-cephalosporins and monobactams (Scheme 1, top); moreover, ESBL-producing organisms are generally resistant to aminoglycosides because the *bla* genes that encode for ESBLs are carried on mobile elements that also carry other antibiotic resistance factors, such as genes encoding aminoglycoside-modifying enzymes. As a result, it is necessary for clinical laboratories to detect the production of ESBLs in order for clinicians to initiate appropriate antibiotic therapy (2). Carbapenems (imipenem, meropenem, ertapenem, and doripenem) are stable against hydrolysis by ESBLs and are regarded as the “drugs of choice” for infections due to ESBL-producing organisms (3,4).

The first plasmid-mediated β -lactamase capable of hydrolyzing extended-spectrum cephalosporins, now known as SHV-2, was reported in 1983 and a number of other groups of β -lactamases with expanded hydrolytic activity were reported thereafter (5,6). The term ‘extended-spectrum β -lactamase’ (ESBL) was applied to denote these enzymes with activity against extended-spectrum cephalosporins, such as cefotaxime, ceftazidime (Scheme 1), ceftriaxone, and cefuroxime (7-9). Sequencing of the encoding genes revealed that most ESBLs described in the 1980s were derived from genes for *bla*_{TEM-1} or *bla*_{SHV-1} by mutations that alter the architecture of the active site. The current emergence of CTX-M type ESBLs, β -lactamases whose substrate profile favors either cefotaxime or ceftazidime hydrolysis, are also becoming an international problem (4,10,11). The latter family of ESBLs is now found in many isolates of *E. coli* found in community acquired urinary tract infections.

In both SHV and TEM backgrounds, the G238S substitution is critical for cefotaxime hydrolysis whereas both the G238S/E240K substitutions are needed for clinically-significant ceftazidime hydrolysis (12,13). Overlay of either the SHV G238S or the TEM G238S crystal structure with the respective WT crystal structure shows a significant 1-3 Å displacement in the 238–242 β -strand-turn segment, which allows the β -lactam binding site to accommodate oxyimino-cephalosporins with large C7 groups, thereby expanding the substrate spectrum of the variant enzyme (14,15). The E240K substitution is only observed in concert with G238S or R164S and confers enhanced ability to hydrolyze ceftazidime and aztreonam, which both possess a bulky 1-carboxy-1-methylethoxyimino side chain (highlighted in Scheme 1). Unlike the G238S substitution, structural data is not available for either TEM or SHV with E240K; however, molecular modeling suggests that the lysine side chain is able to form an electrostatic bond with the carboxylic acid of the oxyimino substituent (highlighted in Scheme 1) (13). If residue 240 is glutamate, as in WT or G238S, its carboxylic acid group encounters the oxime's carboxylic acid group. Consequently, the electrostatic repulsion results in little catalytic activity against either ceftazidime or aztreonam (13).

Another distinguishing and un-explained attribute of ESBLs is their “hyper-susceptibility” to mechanism-based inhibitors, such as sulbactam, tazobactam, and clavulanic acid (Scheme 1, bottom) (16). Following an attack on the β -lactam carbonyl by the S70 side-chain, the resultant acyl-enzyme undergoes a series of proton transfers in which a variety of intermediates, including imine **1**, *cis*-enamine **2**, *trans*-enamine **3**, and irreversible S130-bound acrylate (not shown), are possible (see Scheme 2). Mechanistically, the linear *trans*-enamine intermediate is a long-lived inhibitory species (17-19). Consequently, the “hyper-susceptibility” of SHV-type ESBLs, compared to the WT enzyme, may arise from *relative* or *absolute* differences in the amount of *trans*-enamine formed by a given inhibitor. Previous Raman/X-ray analysis of an inhibitor-resistant β -lactamase showed that addition of the resistant mutation M69V to an E166A background reduced the amount of *trans*-enamine seen with tazobactam and clavulanic acid (20). While significant structural changes were not seen in the binding of the *trans*-

enamine intermediate once it was formed, X-ray crystallography detected a slightly smaller oxyanion hole for the M69V/E166A variant (20). In this work, potential differences in inhibitory population levels are explored in single crystals of SHV-2 or -5 by Raman microscopy, which detects higher relative amounts of the *trans*-enamine acyl-enzyme compared to SHV-1. Additionally, UV spectroscopy in solution shows that, under single turnover conditions, SHV-2 and SHV-5 make approximately 35% more of an enamine-type species than SHV-1. Higher populations of the *trans*-enamine intermediate result in lower K_I values and greater inactivation efficiencies for the mechanism-based inhibitors compared to the WT enzyme. Together, the data explain the observed *in vivo* and *in vitro* “hyper-susceptibility” of ESBLs harboring mutations in the key β -strand of the catalytic site of class A β -lactamases.

Materials and Methods

Bacterial Strains and Plasmids

β -lactamase genes encoding *bla*_{SHV-1}, *bla*_{SHV-2}, and *bla*_{SHV-5} were cloned into the pBC SK (–) phagemid vector (Stratagene, La Jolla, CA) as previously described (21). The β -lactamases were expressed in *E. coli* DH10B cells (Invitrogen, Carlsbad, CA), liberated by stringent periplasmic fractionation, and initially purified by preparative isoelectric focusing (21). The β -lactamases were further purified by size exclusion chromatography using a Waters High-Performance Liquid Chromatography (HPLC) system. The purity of each β -lactamase was assessed on 5% stacking and 12% resolving sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gels were stained with Coomassie Brilliant Blue R250 (Fisher) to visualize the β -lactamases. Protein concentrations were determined by a standard Bradford assay (Bio-Rad) using bovine serum albumin as a standard.

Antibiotic Susceptibility Testing

E. coli DH10B expressing the *bla*_{SHV} genes were phenotypically characterized by lysogeny broth agar dilution minimum inhibitory concentrations (MICs) using a “Steers replicator” that delivered 10^4 colony forming units/spot (21). Concentrations employed for determining MIC values were $\mu\text{g/ml}$. MICs were read after 18–24 h of incubation at 37 °C and performed three times for each antibiotic. Ampicillin, ceftazidime, and cefotaxime were obtained from Sigma-Aldrich Chemical Company. Nitrocefin was obtained from CalBioChem (San Diego, CA). Sodium clavulanate (Smith-Kline-Beecham), sulbactam (Pfizer), and tazobactam (Wyeth Pharmaceuticals) were gifts of the respective companies.

Kinetics

Steady-state kinetics were performed using continuous assays at room temperature in an Agilent 8453 diode array spectrophotometer (Agilent, Palo Alto, CA). Each assay was performed in 10 mM phosphate-buffered saline (PBS) at pH 7.4. Measurements were obtained using nitrocefin (NCF) (BD Biosciences, San Jose, CA) ($\Delta\epsilon_{482} = 17400 \text{ M}^{-1} \text{ cm}^{-1}$).

The kinetic parameters, V_{max} and K_M , were obtained with nonlinear least-squares fit of the data (Henri Michaelis–Menten equation) using Origin 7.5 (OriginLab, Northampton, MA):

$$v = (V_{\text{max}} \times [S]) / (K_M + [S]) \quad (1)$$

For each of the β -lactamase inhibitors, the K_I values (equivalent to K_M) were determined by direct competition assays. The initial velocity was measured in the presence of a constant concentration of enzyme (10 nM) with increasing concentrations of inhibitor against the

indicator substrate, NCF. Care was taken to initiate the reaction with the addition of both NCF and the inhibitor and to limit the initial velocity determination to the first 5 s. The K_I data were corrected to account for the affinity of NCF for SHV-1, SHV-2, and SHV-5 according to the following equation:

$$K_I(\text{corrected}) = K_I(\text{observed}) / [1 + ([S]/K_{M(\text{NCF})})] \quad (2)$$

The first-order rate constant for enzyme and inhibitor complex inactivation, k_{inact} , was obtained by monitoring the reaction time courses in the presence of inhibitor. Fixed concentrations of enzyme (10 nM) and NCF (150 μM) and increasing concentrations of inhibitor were used in each assay. The k_{obs} was determined using a nonlinear least-squares fit of the data using Origin 7.5:

$$A = A_0 + v_I t + (v_0 - v_I) [1 - \exp(-k_{\text{obs}} t)] / k_{\text{obs}} \quad (3)$$

Here, A is absorbance, v_0 (expressed in variation of absorbance per unit time) is initial velocity, v_I is final velocity, and t is time. Each k_{obs} was plotted versus I and fit to determine k_{inact} .

Stopped-flow ultra-violet absorbance analysis was performed on an Applied Photophysics π^* 180 spectrometer. Reactions were performed in 10 mM phosphate-buffered saline (PBS) at pH 7.4. Temperature was regulated at $25.0(\pm 0.1)^\circ\text{C}$ using a circulating water bath. Reactions were initiated by injecting equal volumes of tazobactam and either SHV-1, SHV-2, or SHV-5 into a flow cell. After mixing, the final concentration of both the enzyme and the substrate was 2 μM . Absorbance measurements were recorded at 288 nm using a variable wavelength monochromator and a 75 W mercury-xenon light source.

Crystallization

Initial crystallization screens were carried out using the sitting-drop vapor-diffusion method in 96-well plates. Sitting-drop procedures were carried out by mixing equal volumes of a 5 mg/ml protein solution and crystallizing solution from Crystal Screen 1 & 2 (Laguna Niguel, Hampton Research). Small plates were obtained from solution 20 in Crystal Screen 1 (0.1 M MES pH 6.5, 1.6 M $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$). Following addition of 5.6 mM CYMAL-6 (Hampton Research, Laguna Niguel, CA) to the crystallizing solution, larger crystals were obtained from hanging drops after approximately 2 weeks. The resulting crystals belong to space group $P2_12_12_1$ (personal communication, J. Sampson and F. van den Akker).

Raman Crystallography

The Raman microscope system has been described previously (22). A 647 nm Kr^+ laser beam (Innova 70 C, Coherent, Palo Alto, CA) was focused onto the protein crystals, suspended on the underside of a siliconized quartz coverslip in a 4 μL drop. Laser power (120 mW) was focused using the 20 $\times$ objective to a 20 μm spot on the crystal. The crystals and the laser spot were visualized with video display to ensure alignment with the focal spot of the laser. During data collection, spectra were acquired over 10 s intervals and 10 spectra were averaged for each acquisition time point. Spectra of the apo- β -lactamase protein crystals were obtained, followed by addition of the inhibitors to the drop to achieve a final drop volume of 4 μL and a final inhibitor concentration of 5-10 mM. Spectra were then acquired serially every 2-3 min following addition of inhibitor. An apo- β -lactamase spectrum was subtracted from the inhibited protein spectra at varying time intervals following addition of inhibitor:

$$\text{Difference spectrum} = [\text{protein} + \text{inhibitor}] - [\text{protein}] \quad (4)$$

Data collection and subtractions were performed using HoloGRAMS and GRAMS/AI 7 software (ThermoGalactic, Inc., Salem, NH). Raman spectra of the inhibitors that were used were obtained under similar conditions. Spectra were obtained for 4 μL drops of 100 mM inhibitor solutions prepared in 2 mM HEPES (pH 7.0).

Band Fitting of Raman Spectrum

The conformational heterogeneity of the *trans*-enamine intermediates of tazobactam and clavulanic acid were investigated by analyzing the O=C-C=C-NH stretch of the *trans*-enamine Raman band. The extended enamine region (1575–1645 cm^{-1}) was fitted assuming three component bands that represent a *cis*-enamine band near 1585 cm^{-1} , a *trans*-enamine band near 1600 cm^{-1} , and an antisymmetric stretch of the C3 carboxylate near 1630 cm^{-1} (23). Component bands were fitted by 15–35 cm^{-1} bandwidth features; Gaussian and Lorentzian functions were employed as models of homogeneous and heterogeneous broadening, respectively. Fitting was performed using the Levenberg-Marquardt nonlinear least-squares method as implemented in the CurveFit.Ab routine of GRAMS/32. The baseline was taken as a linear function. The standard error for peak positions is $<1 \text{ cm}^{-1}$ and for peak widths $<2 \text{ cm}^{-1}$ for well-defined components, such as the *trans*-enamine band.

Results and Discussion

Antibiotic Susceptibility

To assess the impact of the amino acid substitutions on the behavior of SHV β -lactamase in *E. coli* DH10B, we first determined the MICs. *E. coli* DH10B cells containing *bla*_{SHV-1} encoded in the pBC SK(-) phagemid vector exhibit robust levels of resistance to ampicillin ($\text{MIC} \geq 16000 \mu\text{g/mL}$) and piperacillin ($\text{MIC} = 2049 \mu\text{g/mL}$). However, for both SHV-2 and SHV-5, ampicillin and piperacillin MICs were decreased by up to four dilutions compared to WT SHV-1 containing *E. coli* strains (Table 1a).

For *E. coli* expressing SHV-1 β -lactamase, MICs for cefotaxime (0.25 $\mu\text{g/mL}$) and ceftazidime (2 $\mu\text{g/mL}$) were similar to control cells (Table 1b). While both the G238S and G238S/E240K variants conferred significantly higher levels of resistance to cefotaxime than the parent enzyme ($\text{MIC} = 16$ and $32 \mu\text{g/mL}$, respectively), only the G238S/E240K double substitution resulted in high-level resistance to ceftazidime ($\text{MIC} \geq 128 \mu\text{g/mL}$).

The results shown in Table 1b confirm that, while SHV-2 and SHV-5 have activity towards cefotaxime and ceftazidime, they are also “hyper-susceptible” to the clinical inhibitors sulbactam, tazobactam, and clavulanic acid. Currently, the use of β -lactam antibiotics in combination with β -lactamase inhibitors represents a potentially effective measure to combat a specific resistance mechanism of some β -lactamase producing organisms. Compared to the WT-producing strain, *E. coli* DH10B containing either SHV-2 or SHV-5 were more susceptible to β -lactam/ β -lactamase inhibitor combinations. For both ESBL variants, susceptibility to these combinations was reduced by one dilution for ampicillin/clavulanic acid ($\text{MIC} = 50/1 \mu\text{g/mL}$), four dilutions for ampicillin/sulbactam ($\text{MIC} = 50/16 \mu\text{g/mL}$), and five dilutions for ampicillin/tazobactam ($\text{MIC} = 50/8 \mu\text{g/mL}$) compared to wild-type (Table 1c).

Kinetic Behavior of “Hyper-Susceptible” β -Lactamases with the Mechanism-Based Inhibitors

Next, we show that the observed decrease in antibiotic susceptibility of *E. coli* cells containing either *bla*_{SHV-2} or *bla*_{SHV-5} to tazobactam, sulbactam, and clavulanic acid results from only minor changes in the steady-state kinetic parameter (K_I , k_{inact} , and K_I/k_{inact}). A common feature of the “hyper-susceptible” enzymes SHV-2 and SHV-5 is a decrease by less than one order of magnitude for clavulanate and tazobactam and 20- to 50-fold for sulbactam in the K_I values for the mechanism-based inhibitors (Table 2). With clavulanate, both the WT and “hyper-susceptible” enzymes exhibited similar k_{inact} values. Consequently, the decreased K_I of these variant enzymes for clavulanate is solely responsible for their increased inactivation efficiency (k_{inact}/K_I). In contrast, the WT enzyme exhibited an increased k_{inact} value with tazobactam compared to SHV-2 and SHV-5; however, the inactivation efficiency (k_{inact}/K_I) of the extended-spectrum β -lactamases was still increased slightly over the wild-type value (Table 2). Sulbactam was rapidly hydrolyzed by both the WT and extended-spectrum β -lactamases. As a result, values for k_{inact} were not able to be determined. Despite the increased inactivation efficiencies of β -lactamase inhibitors against SHV-2 and SHV-5, the effects are less than one order of magnitude. Because sulbactam, tazobactam, and clavulanate follow a multistep reaction pathway, we next examined the relative populations of the inhibitory intermediates formed with each β -lactamase to explain the kinetic data and antibiotic susceptibilities.

Clinical Inhibitors Form a Predominant Population of the *trans*-Enamine with SHV-2 and SHV-5

In the following sections, we present evidence using Raman crystallography that, compared to wild-type SHV-1, the clinical inhibitors form measurably higher populations of the hydrolytically-inert *trans*-enamine with the ES β -lactamases, SHV-2 and SHV-5. Moreover, we show that the *trans*-enamine acyl-enzymes of ES β -lactamases are held in a rigid and non-flexible conformation, which may increase their stability and which can be attributed, at least in part, to a conformational change of the 238-242 β -strand segment. The Raman difference spectra of the clinical inhibitors with a deacylation deficient mutant E166A and the wild-type enzyme are provided for comparison. For brevity, only the SHV-2 difference spectra are shown for comparison. The SHV-5 difference spectra are nearly identical to those of SHV-2 and appear in the Supplementary Material.

Sulbactam

The difference spectra of the intermediate acyl-enzyme populations of sulbactam with E166A, SHV-1, and SHV-2 are shown in Figure 1a-c. The Raman spectra of the *trans*-enamine species formed by sulbactam with E166A β -lactamase is shown in Figure 1a and discussed at length in Helfand *et al* (17). A predominant *trans*-enamine species is identified in the active site, with a Raman signature peak near 1600 cm^{-1} from the stretching motions of the $\text{O}=\text{C}-\text{C}=\text{C}-\text{NH}$ moiety. There are minor contributions in the E166A:sulbactam difference spectra from both the imine (1654 cm^{-1}) and *cis*-enamine, which displays a shoulder near 1590 cm^{-1} . In previous studies, we determined that the maximum amount of *trans*-enamine intermediate was attained within 20-30 minutes, which permitted us to identify the conditions needed for optimizing the *trans*-enamine populations in single crystals for flash freezing and for colleagues to obtain high-resolution crystal structures (17-19). The SHV-1:sulbactam difference spectra is shown in Figure 1b. Unlike E166A, it is immediately obvious that sulbactam does not form a predominant population of *trans*-enamine with the wild-type enzyme. The conjugated $\text{O}=\text{C}-\text{C}=\text{C}-\text{NH}$ stretch of the *trans*-enamine at 1606 cm^{-1} and a peak of similar intensity at 1588 cm^{-1} , which is assigned to the $\text{O}=\text{C}-\text{C}=\text{C}-\text{NH}$ stretch of the *cis*-enamine, indicate nearly equal populations from the *cis*- and *trans*-enamine acyl-enzymes at steady state. The *cis/trans*-enamine vibrations couple with the asymmetric $-\text{CO}_2$ stretch to give an unresolved medium-

intensity band near 1630 cm^{-1} . Additionally, the 1654 cm^{-1} band is assigned to the $\text{C}=\text{NH}^+$ stretch of the imine acyl-enzyme. Thus, with sulbactam, SHV-1 difference spectra show evidence for a mixture of *trans*- and *cis*-enamine and an imine population where, compared to E166A, there is a relatively smaller amount of *trans*-enamine. Deuterium isotope exchange experiments, *ab initio* calculations, and model systems that were used to assigned the *cis*-enamine and imine bands are presented in Kalp *et al* (23). Lastly, the intermediate acyl-enzyme populations of sulbactam with the “hyper-susceptible” variant, SHV-2, are shown in Figure 1c. With the exception of the intense doublet at 1677 and 1663 cm^{-1} , the spectrum closely resembles the E166A:sulbactam difference spectra. The band seen at 1554 cm^{-1} is due to a tryptophan ring mode. Again, a predominant population of the *trans*-enamine species is identified in the active site, with a Raman signature peak at 1601 cm^{-1} . Compared to SHV-1, the Raman difference spectra of the acyl-enzymes formed between sulbactam and SHV-2 or E166A show a relative increase in the *trans*-enamine population. This indicates that SHV-2 and E166A selectively stabilize the *trans*-enamine and slow its tautomerization to the hydrolysis-prone imine. The resonance-stabilized vinylogous urethane is common to both the *cis*- and *trans*-enamine and deactivates them to hydrolytic deacylation; however, *cis* to *trans* isomerization is favored to due to the intrinsic steric clash of the *cis* form. Isomerization occurs via the common imine, which is hydrolytically labile due to lack of conjugation with the acyl carbonyl group. As a result, enzymes, such as SHV-1, that have a major population of *cis*-enamine (and thus imine) are not as effectively inhibited by sulbactam. This is reiterated by both the MIC values (Table 1c) and the kinetic data (Table 2).

Clavulanic Acid

As we have reported previously, the *trans*-enamine is the sole intermediate acyl-enzyme formed when clavulanic acid is reacted with E166A β -lactamase crystals (17). A high-resolution structure confirmed that the *trans*-enamine of clavulanic acid was bound to E166A with nearly 100% occupancy (18). Accordingly, the Raman spectrum of this species demonstrates a single band at 1602 cm^{-1} (Figure 2a). Analysis of our previously published Raman difference spectra indicated that all three inhibitors, free in solution, have a medium intensity Raman mode near 1400 cm^{-1} , which is due to the symmetric stretching vibration of the $-\text{CO}_2^-$ group (17,23). For the bound tazobactam and sulbactam, this feature shifts to about 1375 cm^{-1} , reflecting electrostatic interactions of the $-\text{CO}_2^-$ with active site residues. For clavulanic acid bound to E166A, however, there was no peak in this region, which suggested that the $-\text{CO}_2^-$ group was no longer present (17). This observation was also confirmed by X-ray crystallography (23). When clavulanic acid is reacted with the wild-type enzyme, two additional bands are noted in the difference spectrum at 1662 and 1647 cm^{-1} (Figure 2b). These bands have been assigned to a tautomer pair of imine acyl-enzymes (Scheme 3) (23). The 1662 cm^{-1} band in the SHV-1:clavulanate complex is assigned to a $\text{C5}=\text{N4}$ imine that results from cleavage of the $\text{C}-\text{O}$ bond and concomitant opening of the oxazolidinium ring. Structurally, this imine is analogous to those formed by sulbactam and tazobactam. Tautomerization of the $\text{C5}=\text{N4}$ imine to the $\text{N4}=\text{C3}$ imine is driven by resonance with the adjacent ketone at C2. The $\text{N4}=\text{C3}$ imine, which is marked by the 1647 cm^{-1} band in the Raman spectrum, is unique to clavulanate and was first detected by mass spectrometry (24). Assignments of the imine bands, reported in Kalp *et al* (23), are based on their frequency in the Raman spectra, QM calculations, response to NH/ND exchange, and response to reduction by sodium cyanoborohydride, a reducing reagent that is specific for protonated imines(23). In addition, clavulanate may generate a minor population of *cis*-enamine; like sulbactam, the 1585 cm^{-1} shoulder features of both the E166A and SHV-1 difference spectra are assigned to *cis* $\text{O}=\text{C}-\text{C}=\text{C}-\text{NH}$ stretch. The clavulanate:SHV-2 difference spectrum, shown in Figure 2c, is similar to the wild-type difference spectrum with one notable exception: Clavulanic acid forms a greater amount of *trans*-enamine relative to imine with SHV-2 than with SHV-1. Because the *trans*-enamine is responsible for transient inhibition of the enzyme, a β -lactamase that stabilizes this

intermediate, such as SHV-2, will be more susceptible to mechanism-based inhibitors than a β -lactamase, such as SHV-1, which forms relatively more of the hydrolysis-prone imine.

Tazobactam

Tazobactam possesses superior *in vivo* and *in vitro* efficacy against SHV β -lactamases when compared to sulbactam or clavulanic acid. Unlike the other inhibitors, tazobactam forms a predominant population of *trans*-enamine with **both** SHV E166A and WT SHV-1 β -lactamases. In the Raman difference spectra, the O=C-C=C-NH stretch of the *trans*-enamine appears as a relatively intense band at 1593-1596 cm^{-1} (Figure 3). One notable exception between the E166A and wild-type difference spectra is the presence of a sharp band at 1658 cm^{-1} in the latter. This band is assigned to the C=NH⁺ stretch of a protonated imine species and represents a population of hydrolysis-prone acyl-enzymes. There is little if any contribution from the C=NH⁺ stretch of the imine to the tazobactam:E166A difference spectrum. The tazobactam-bound difference spectrum obtained with SHV-2 is shown in Figure 3c. Aside from bands at 1663 and 1677 cm^{-1} that result from protein conformational changes, the spectrum is very similar to those presented in Figure 3a and b. One small difference is the decrease intensity of the imine's C=NH⁺ stretch at 1658 cm^{-1} in the tazobactam:SHV-2 difference spectrum (Fig. 3c) compared to WT SHV-1 (Fig. 3b). Although this change is difficult to observe due to the surrounding interference from the protein bands, it is reproducible. In addition to changes in imine population, there is another factor that may operate to provide the “hyper-susceptibility” of ES β -lactamases to mechanism-based inhibitors.

Next, we present evidence that SHV-2 and SHV-5 form *trans*-enamine intermediates in potential minima which are more well-defined than their ‘floppy’ counterparts in SHV-1. This evidence comes from a comparison of the *trans*-enamine peak widths of tazobactam and clavulanic acid in several different SHV β -lactamases: the deacylation-deficient variant, SHV E166A; two ESBLs, SHV-2 and SHV-5; and wild-type SHV-1 at 4 °C and room temperature. After analyzing the individual difference spectra at 20 min., the following order of increasing *trans*-enamine bandwidths at half-height was found for both tazobactam and clavulanic acid: SHV E166A < SHV-2 < SHV-5 < SHV-1 (4 °C) < SHV-1 (Figure 4). In the Raman spectroscopic studies of biological molecules, broadened band shapes are usually attributed to static and/or dynamic conformational heterogeneity. In a recent study, a strong correlation was observed between Raman bandwidth and X-ray B-factors (18). Thus, in the present instance, the broader peak widths in Figure 4 of the tazo-derived fragments from SHV-1 are ascribed to the existence of multiple forms differing slightly in conformation. Chemically, these have the same parent *trans*-enamine structure; however, a range of slightly different torsional angles in the enamine skeleton effectively broadens the band profile compared to the same feature in the SHV-2 and -5 difference spectra (Figure 4). A second possibility relates to the inhibitor “tails” extending beyond N4. Calculations confirm that extensive vibrational coupling exists between motions in the O=C-C=C-NH fragment and adjacent bonds. Thus, static or dynamic disorder in the tail can also lead to broadening of the band near 1600 cm^{-1} . Regardless, the effect of stabilizing the *trans*-enamine intermediate is to reduce the rate constant for tautomerization back to the hydrolysis-prone imine. The Raman data show that the *trans*-enamine intermediates of SHV-2 and -5 are in well-defined conformations that undergo only minimal excursions from the mean dihedral angle; and we hypothesize that this increases the intermediate's stability. This trend explains the *in vivo* and *in vitro* responses of these ESBLs to tazobactam and clavulanic acid. Unfortunately, a simple band shape analysis is not possible for sulbactam due to the spectral complexity as seen in Figure 1.

Protein Conformational Change

In the difference spectrum of each inhibitor with the extended-spectrum variant SHV-2, the intense features near 1663 and 1678 cm^{-1} are assigned to protein amide I modes that appear

due to secondary structural changes occurring upon acylation (Figure 1c, 2c, and 3c). The protein bands are less apparent in the clavulanate:SHV-2 difference spectrum because of interference from ligand-associated modes. In the spectrum of native crystals, the amide I profile, 1620-1700 cm^{-1} , contains contributions at different positions from modes due to α -helices, β -sheets, β -strands, and unordered regions of polypeptide. When β -sheet or β -strand features appear in the difference spectra, such as the Raman lines between 1663 and 1676 cm^{-1} , respectively, it indicates a ligand-induced conformational change involving β -structure. SHV-2 contains a single Gly238Ser change relative to the wild-type enzyme SHV-1; thus, any additional protein conformation changes that occur from binding the inhibitor to SHV-2 compared to SHV-1 must be attributed to this single amino acid substitution. A high-resolution crystal structure of SHV-2 shows a significant 1-3 Å displacement in the 238-242 β -strand-turn segment compared to SHV-1. The Gly238Ser mutation makes this β -strand, which forms one wall of the substrate binding site, more flexible. Consequently, the binding site is more open to newer cephalosporins with large C7 substituents and thereby expands the substrate spectrum of the variant enzyme. We propose that the conformational changes observed in the SHV-2 and SHV-5 difference spectra represent a tightening of the β -structure in the 238-242 β -strand-turn segment that occurs upon acylation by a mechanism-based inhibitor. In turn, this creates a more rigid binding site for the inhibitor and drives the *trans*-enamine intermediate to adopt a more rigid conformation. When oxyimino-cephalosporins, such as cefotaxime and ceftazidime, bind to SHV-2 or SHV-5, the flexible β -strand-turn segment is held in an open conformation to accommodate the large C7 substituents. Thus, we do not observe a conformational change as we do for the small clinically relevant inhibitors. The absence of a conformational change giving rise to features in the 1663-1678 cm^{-1} region is apparent in Figure 5, which shows the Raman difference spectra of SHV-2 with cefotaxime and ceftazidime following a 5 minute soak.

Detection of Enamine by Stopped-Flow Ultraviolet Spectroscopy

There are two general findings from the Raman data: 1) compared to WT, SHV-2 and -5 form relatively more *trans*-enamine (when compared to the relative population levels of imine and *cis*-enamine); and 2) the *trans*-enamine intermediate of tazobactam in SHV-2 and SHV-5 is in a 'tighter' conformation than in SHV-1. Additional support for conclusion (1) comes from stopped-flow kinetic studies.

Under pre-steady state conditions, UV time traces were recorded between 1:1 complexes of tazobactam and SHV-1, -2, or -5 β -lactamase. The change of absorbance at 288 nm over 0-10 s is shown for each enzyme in Figure 6. An increase in absorbance at 288 nm is indicative of the formation of an enamine-type intermediate (25). The λ_{max} and extinction coefficient of the enamine chromophore do not differ significantly between SHV-1, -2, or -5 (unpublished data, Kalp and Carey); consequently, the traces can be used to compare the amount of enamine formed among the various enzymes. As shown in Figure 6, SHV-2 and -5 form approximately 35% more of the enamine-type intermediate than SHV-1. Studies on the β -lactamase (EC 3.5.2.6) from *Staphylococcus aureus* attributed the biphasic nature of the traces to a *cis*- to *trans*-isomerization, whereby the first-formed enamine with a UV maximum near 290 nm converts to a stable form of the inactivated enzyme that has a minimum at 275 nm (26,27). Because the Raman spectra are acquired on the time scale of minutes, the reported isomerization would be near completion by the acquisition of the first Raman spectrum at 2 minutes.

Conclusion

Mention of inhibitor "hyper-susceptibility" is widespread throughout the basic and clinical science literature on ESBLs; however, studies to explain this phenomenon are lacking. Using

two of the most prevalent SHV-type ESBLs, SHV-2 and SHV-5, we show that “hyper-susceptibility” to tazobactam, sulbactam, and clavulanic acid is the direct result of increased stability of the *trans*-enamine intermediate in ESBLs compared to WT. The increased stability, as detected by Raman band shape analysis of the *trans*-enamine's O=C-C=C-NH stretch, slows tautomerization to the hydrolytically-labile imine and leads to increased populations of the *trans*-enamine acyl-enzyme as determined by both Raman difference spectroscopy and time-resolved UV spectroscopy. As a result, the K_I , or the inhibitor concentration required to produce half maximum inhibition, is lowered by as much as 50-fold for the mechanism-based inhibitors. While these changes to the kinetic parameters are small, they are sufficient to produce “hyper-susceptibility” as judged by the MICs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ESBL	extended-spectrum β -lactamase
WT	wild-type

TEM	class A β -lactamase of <i>E. coli</i> first described in a Greek patient, with the name being derived from the patient's name
SHV	class A β -lactamase of <i>K. pneumoniae</i> initially thought to be a “sulfhydryl variant” of the TEM enzyme
MES	2-(4-morpholino)ethanesulfonic acid
HPLC	high-performance liquid chromatography
MIC	minimal inhibitory concentration

Figure 1. Partial Raman difference spectra of sulbactam reacting in crystals of three β -lactamases: a) SHV E166A, b) wild-type SHV-1, and c) SHV-2, which carries the G238S substitution.

Figure 2. Partial Raman difference spectra of clavulanic acid reacting in crystals of three β -lactamases: a) SHV E166A, b) wild-type SHV-1, and c) SHV-2.

Figure 3. Partial Raman difference spectra of tazobactam reacting in crystals of three β -lactamases: a) SHV E166A, b) wild-type SHV-1, and c) SHV-2.

Figure 4.

Peak widths at half height for the intense *trans*-enamine stretching feature near 1600 cm⁻¹ for clavulanic acid and tazobactam reacting in single crystals of the named enzymes. The complex spectral profile precludes measurements for sulbactam.

Figure 5. Partial Raman difference spectra of cefotaxime (top) and ceftriaxone (bottom) reacting in single crystals of SHV-2. Note the lack of features from 1660-1680 cm^{-1} , which indicates that these compounds do not bring about a conformational change in the active site.

Figure 6. Stopped-flow absorption traces for tazobactam reacting with WT SHV-1, SHV-2 and SHV-5 in equimolar ratios. Note higher absorbance at 288 nm at ~1 second indicates higher enamine populations for SHV-2 and SHV-5.

Scheme 1.
No caption.

Scheme 2.

Reaction scheme for sulbactam and serine-70 in the active site of a β -lactamase. “1” is an imine, and “2” and “3” are *cis*- and *trans*-enamines, respectively. In addition to deacylation the imine (1) can be attacked by S130 to yield an acrylate-like species.

Scheme 3.

The imine tautomers formed after acylation of the active-site serine by clavulanic acid.

Table 1

MICs for the DH10B strain alone and producing SHV-1, SHV-2 (G238S), or SHV-5 (G238S/E240K).

a.

	Ampicillin (µg/ml)	Piperacillin (µg/ml)
DH10B	1	2
SHV-1	>16,382	2048
SHV-2	8192	512
SHV-5	16384	512

b.

	Ceftazidime (µg/ml)	Cefotaxime (µg/ml)
DH10B	1	0.25
SHV-1	2	0.25
SHV-2	8	16
SHV-5	>128	32

c.

	Ampicillin/ Tazobactam (µg/ml)	Ampicillin/ Sulbactam (µg/ml)	Ampicillin/ Clavulanic Acid (µg/ml)
DH10B	1	1	1
SHV-1	128	512	2
SHV-2	8	16	1
SHV-5	8	16	1

Table 2

Steady-state kinetic parameters.

a) Due to the rapid hydrolysis of sulbactam by both the WT and extended-spectrum β -lactamases, values for k_{inact} were not able to be determined. Consequently, the K_i values rather than K_I values are given. See Materials and Methods for further details.

	ClavulanicAcid	Sulbactam ^a	Tazobactam
$K_i(\mu\text{M})$			
SHV-1	1 ± 0.04	8.6 ± 0.7	0.200 ± 0.035
SHV-2	0.330 ± 0.030	0.490 ± 0.050	0.030 ± 0.003
SHV-5	0.158 ± 0.013	0.200 ± 0.016	0.019 ± 0.001
$k_{\text{inact}}(\text{s}^{-1})$			
SHV-1	0.04 ± 0.02		0.14 ± 0.01
SHV-2	0.05 ± 0.01		0.030 ± 0.003
SHV-5	0.06 ± 0.01		0.025 ± 0.001
$k_{\text{inact}}/K_i(\mu\text{M}^{-1}\text{s}^{-1})$			
SHV-1	0.04 ± 0.03		0.70 ± 0.12
SHV-2	0.15 ± 0.03		1.00 ± 0.14
SHV-5	0.38 ± 0.07		1.32 ± 0.10