Avibactam and inhibitor resistant SHV β-lactamases

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Abstract:

β-Lactamase enzymes (E.C.3.5.2.6) are a significant threat to the continued use of β-lactam antibiotics to treat infections. A novel non-β-lactam β-lactamase inhibitor, avibactam, is being developed with activity against many β-lactamase variants. Here, we explore the activity of avibactam (a diazabicyclocloctane) against bacteria containing a variety of characterized isogenic laboratory constructs of β-lactamase inhibitor resistant variants of the class A enzyme SHV. We find that the S130G variant of SHV shows significant resistance to inhibition by avibactam by both microbiological and biochemical characterization. Our analysis leads us to hypothesize that the lack of a hydroxyl group at the 130 position in the S130G variant of SHV-1 slows carboxylation of the enzyme by avibactam. Thus, the S130G substitution is a common “key residue” for the inhibition of class A β-lactamases, perhaps even for the novel diazabicycloctane class of β-lactamase inhibitors.

Introduction:

β-Lactam antibiotics (penicillins, cephalosporins, and carbapenems) are a cornerstone of therapy for many Gram-negative bacterial infections. These antibiotics act by binding to the penicillin-binding proteins of the bacterial cell membrane, preventing peptidoglycan cross-linking and eventually leading to cell lysis and death. Recent studies also shown that penicillin creates a “futile cycle” involving a lytic transglycosylase (Slt) leading to bacterial cell death (1).

Unfortunately, Gram-negative bacteria contain β-lactamase enzymes in their periplasmic space that are able to hydrolyze the amide bond of β-lactams preventing these compounds from reaching their site of action. In order to combat the action of these enzymes, the synthesis of additional classes of β-lactams (i.e. future generations of cephalosporins, carbapenems, monobactams) and the synthesis of β-lactamase inhibitors (BLIs) (i.e. sulbactam, tazobactam,
and clavulanic acid) was undertaken. Despite the clinical success of β-lactams and BLIs, β-lactamase enzymes have evolved to exhibit resistance to both of these strategies; point mutations leading to extended-spectrum β-lactamases (ESBLs, enzymes resistant to later-generation cephalosporins) and inhibitor resistant (IR) β-lactamases (enzymes which exhibit resistance to inactivation, particularly by clavulanic acid) are the major mechanism by which resistance is manifested.

The SHV-1 β-lactamase is a class A enzyme that is chromosomally encoded in Klebsiella pneumoniae. SHV-1 typically acquires ESBL activity with a substitution at Ambler position Gly-238 or a Glu-240 (2, 3). In contrast, IR variants typically possess substitutions at Ambler position Met-69, Ser-130, Lys-234, Arg-244, or Asn-276 (3). Many of these IR variants have appeared clinically (Table 1) indicating that substitutions at these amino acids pose a significant threat to our current commonly prescribed β-lactam/BLI combinations (amoxicillin-clavulanate (Augmentin™), piperacillin-tazobactam (Zosyn™), and ampicillin-sulbactam (Unasyn™)) (3).

The inactivation of a β-lactamase enzyme by a BLI (such as tazobactam, sulbactam, or clavulanic acid) involves an attack by the active site serine (in the case of SHV, Ser-70) on the amide bond, formation of the acyl-enzyme, and then subsequent rearrangement steps leading to an imine and enamine and either transient or long-lived inhibition of the enzyme (Figure 1A) (4). In order to understand this mechanism of inhibition, mutagenesis studies have been performed with amino acid substitutions in the SHV-1 β-lactamase that lead to resistance to inactivation by clavulanic acid. Notably, many of these IR β-lactamase variants are hypothesized to have a common mechanism involving Ser-130 which prevents or slows permanent inhibitor inactivation of the enzyme (5-14).
Avibactam is a novel non-β-lactam BLI that was tested in Phase III clinical trials in combination with ceftazidime (ceftazidime-avibactam) evaluating its efficacy against bacteria causing complicated intraabdominal infections, complicated urinary tract infections, and nosocomial pneumonia (NCT01644643, NCT01499290, NCT01808092, NCT01599806, NCT01595438, NCT01726023, NCT01500239). Avibactam is a diazabicyclooctane (DBO) inhibitor that does not have a β-lactam ring at the core of its structure. DBOs form a reversible inhibitory reaction that proceeds first through an acyl (carbamyl) enzyme and then undergoes recyclization of the active ring structure so decarbamylation can occur. Avibactam inactivation of class A β-lactamase enzymes is proposed to involve similar amino acid residues compared to the β-lactam-based inhibitors (Figure 1).

We sought to understand the efficacy of this novel DBO inhibitor against a variety of SHV enzymes with known IR amino acid substitutions to determine if avibactam is able to inactivate β-lactamases that are resistant to clavulanic acid. Our studies begin to explore the mechanism of carbamylation and recyclization of avibactam against the class A β-lactamase, SHV-1, to identify the role of important amino acid residues involved in avibactam inactivation activity of this class A β-lactamases, and to anticipate resistance patterns that may be observed by existing variants of SHV-1 once this drug enters the clinic. This information is essential as derivatives of avibactam and future DBO BLIs continue to be developed.

Materials and Methods:

Mutagenesis:
The tested IR SHV *bla* genes were directionally subcloned into the pBC SK(-) vector and have all been previously published (7, 11-14).

Minimum Inhibitory Concentration (MIC) measurement:
MICs were performed by the agar dilution method according to the Clinical Laboratory and Standards Institute (CLSI) protocol (15). Briefly, bacterial cultures were grown overnight at 37°C in Mueller-Hinton (M-H) broth. The cultures were diluted and a Steers™ replicator was used to stamp 10 µL of each dilution onto Mueller-Hinton agar plates. Plates were incubated overnight at 37°C and the MIC was read as the β-lactam concentration at which bacterial growth was no longer observed. We tested ampicillin (AMP, Sigma-Aldrich), AMP-avibactam (AVI), AMP-sulbactam (SUL, Astatech), AMP-clavulanic acid (CLAV, USP), piperacillin (PIP, Sigma-Aldrich), and PIP-tazobactam (TAZO, Chem-Impex). AVI, SUL, and CLAV were added to plates at a constant concentration of 4 mg/L with increasing concentrations of AMP. PIP-TAZO was maintained at a constant 8:1 ratio. Structures of the tested antibiotics are shown in Figure 1A.

Protein purification:

SHV-1 and SHV S130G β-lactamase were purified from E. coli DH10B cells containing bla_SHV-1 or bla_SHV_S130G on a pBC SK (-) plasmid as previously described (16). Briefly, cells were grown overnight in super optimal broth (SOB) at 37°C containing chloramphenicol for plasmid maintenance. After harvesting, cell pellets were pelleted and frozen at -20°C. After 48 hours the pellets were thawed and resuspended in 50 mM Tris-HCl, pH 7.4. Lysozyme (40 µg/mL), benzonase nuclease, MgSO₄ and 1 mM EDTA were added as previously described (16). The cellular debris was then removed by centrifugation and the lysate was separated overnight using preparative isoelectric focusing (pIEF). Regions of the pIEF gel demonstrating the ability to hydrolyze NCF were then further purified by gel filtration (GF) column chromatography on an Äkta fast protein liquid chromatography (FPLC) system to > 95% purity by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The protein concentrations were...
measured using the absorbance at $\lambda = 280$ nm and the extinction coefficient of each protein ($\varepsilon$) calculated using the ProtParam tool at Expasy (http://us.expasy.org/tools).

$\beta$-lactamase kinetics:

The kinetic constants of SHV-1 and S130G $\beta$-lactamases for the hydrolysis of nitrocefin (NCF, Becton, Dickinson, and Company), $K_m$ and $k_{cat}$, were performed according to previously published methods (7). Briefly, an Agilent 8453 diode spectrophotometer was used to measure the absorbance of the hydrolysis of NCF at $\lambda = 482$ nm ($\varepsilon = 17,400$ M$^{-1}$cm$^{-1}$). $\beta$-lactam concentration was held constant at 6 nM for SHV-1 and 390 nM for SHV S130G while the concentration of NCF was varied. The data were plotted and the $K_m$ for NCF was calculated using the Michaelis-Menten equation and Enzfitter™ (Biosoft Corporation, Ferguson, MO) described below as Equation 1:

\[ v = \frac{v_{max} \cdot [S]}{K_m + [S]} \]  
\text{Eq. 1}

Inhibitory enzyme kinetics were performed on the purified SHV-1 and SHV S130G proteins in the same concentrations as described above. These experiments are described below.

The proposed interaction scheme of a class A $\beta$-lactamase and avibactam is shown below as Equation 2 (17).

\[
\begin{align*}
E + I & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} E: I \\
& \overset{k_2}{\longrightarrow} E-I
\end{align*}
\]  
\text{Eq. 2}

The inhibition constant $K_{i, app}$ was measured by a direct competition assay between NCF and avibactam at 25 °C in 10 mM phosphate-buffered saline (PBS). NCF was held constant at 100 µM and the concentration of avibactam was varied. Absorbance was measured on an Agilent 8453 diode spectrophotometer (Santa Clara, CA) as previously described (18). In this assay, SHV S130G $\beta$-lactamase was used at a concentration of 390 nM and SHV-1 was tested at a
concentration of 6 nM. A higher concentration of S130G β-lactamase was required for all experiments due to the lower catalytic efficiency of NCF hydrolysis (7). Excel was used to plot the inverse initial steady-state velocity (1/vi) vs avibactam concentration [I] and the value of the y-intercept divided by the slope of the line was defined as the $K_{i,\text{app\,obs}}$. This value was corrected for the affinity of NCF by the enzyme according to Equation 3 to obtain the $K_{i,\text{app}}$.

$$K_{i,\text{app}} = \frac{K_{i,\text{app\,obs}}}{1 + \frac{[I]}{K_{m,\text{NCF}}}} \quad \text{Eq. 3}$$

$k_2/K$ was determined using timed-inactivation curves for SHV-1 and the S130G variant using NCF as a reporter substrate. SHV S130G was used at a concentration of 98 nM and SHV-1 was used at a concentration of 6 nM. A $k_{\text{obs}}$ value was determined for each avibactam concentration using Origin 3.5.1 according to equation 4.

$$y = V_f \ast x + (V_1 - V_f) \ast \frac{1 - e^{-k_{\text{obs}} \ast x}}{k_{\text{obs}}} + A_l \quad \text{Eq. 4}$$

The $k_{\text{obs}}$ values were then plotted against the avibactam concentration to obtain a straight line. Equation 5 was then used to determine $k_2/K$, the second-order rate constant for enzyme acylation.

$$k_{\text{obs}} = k_2 + \frac{k_2}{K} \ast \frac{[I]}{1 + \frac{[I]}{K_{m,\text{NCF}}}} \quad \text{Eq. 5}$$

The observed $k_2/K$ was then corrected for the affinity of nitrocefin according to equation 6 to obtain the corrected $k_2/K$ value.

$$\frac{k_2}{K_{\text{corr}}} = \frac{k_2}{K} \ast \frac{[I]}{K_{m,\text{NCF}}} + 1 \quad \text{Eq. 6}$$

The partition ratio or turnover number ($k_{\text{cat}}/k_{\text{inact}}$, $t_n$) was determined for each enzyme by incubating at various inhibitor:enzyme ratios during a 24 hour time period. Then, an aliquot was removed and the hydrolysis of NCF was measured. The ratio of avibactam:β-lactamase required to inhibit NCF hydrolysis by > 90% vs. an uninhibited control was defined as the $k_{\text{cat}}/k_{\text{inact}}$ as previously described (18).
Electrospray Ionization (ESI) Mass-Spectrometry (MS):

A Waters Synapt G2-Si High Resolution quadrupole time-of-flight mass spectrometer (Waltham, MA) equipped with a LockSpray dual electrospray ion source was used to acquire mass-spectrometry spectra for purified protein and protein-avibactam complexes in order to determine the nature and timeline of avibactam inactivation of SHV-1 and SHV S130G. Glu-1-fibrinopeptide B was used as the lockmass and the Synapt G2-Si was calibrated with sodium iodide using a mass range of 50-2000 m/z. This calibration resulted in an error of ± 1 amu. After protein purification, and/or incubation with avibactam, 0.2% formic acid was added to quench any reactions. SHV-1 and SHV S130G proteins were incubated (at 30 µM) with a 1:1 ratio of avibactam:protein for various time points, 1 min, 5 min, 6 min, 9 min, 12 min, 15 min, 18 min, 1 hr, and 24 hr at room temperature in 10 mM PBS pH 7.4. C18 Zip-Tips were used to remove residual salt and to concentrate the samples according to the manufacturer’s protocol (Millipore, Billerica, MA). Then, direct infusion at a rate of 50 µL/min was used to perform mass-spectrometry analysis on the eluted protein sample diluted into 50% acetonitrile and 0.2% formic acid. Data were collected for 1 min. Lock mass spectra were collected prior to each sample in a similar manner. The tune settings for each data run were as follows: capillary voltage at 3.2kV, sampling cone at 30, source offset at 30, source temperature at 100°C, desolvation temperature at 450°C, cone gas at 50 L/h, desolvation gas at 600 L/h, and nebulizer bar at 6.0. Spectra were analyzed using MassLynx v4.1. Spectra were modified for lock mass deviations by applying a gain factor and then deconvoluted using the MaxEnt1 program.

Molecular modeling:

The crystal structures of SHV-1 (PDB ID: 1SHV) and SHV S130G (PDB ID: 1TDL) β-lactamases were used to construct and validate Michaelis-Menten and acyl-enzyme complexes of
SHV-1 with avibactam using Discovery Studio (DS) 3.1 molecular modeling software (Accelrys Inc., San Diego, CA) as previously described (11, 18). Avibactam was first constructed using the Fragment Builder tools and was minimized using a Standard Dynamics Cascade protocol of DS 3.1. Avibactam was then automatically docked into the active sites of SHV-1 and SHV S130G using the CDOCKER module of DS 3.1. This protocol uses a CHARMM-based molecular dynamics scheme to dock ligands into a receptor binding site. The best conformations were automatically aligned into polar and apolar active-site “hotspots” and the best scoring poses were reported and hydrogen atoms were not maintained. To further optimize the docked poses (by adding hydrogen atoms and preventing steric clashes between the receptor and ligand) a CHARMM-minimization step was used. In this step, the Smart Minimization algorithm was utilized (1000 steps of Steepest Descent with a RMS gradient tolerance of 3 Å followed by Conjugate Gradient minimization with a root-mean-squared deviation (RMSD) minimization gradient of 0.001 Å). For the last minimization of the avibactam conformations into the active sites of SHV-1 and SHV S130G β-lactamases, a RMSD cut-off of 1 Å was chosen.

The resulting conformations of the SHV-1-avibactam and SHV S130G-avibactam complexes were analyzed and the most favorable positioning of avibactam was chosen. Then, the complexes between the enzymes and inhibitor were created (11, 18). To check the stability of the complexes, an 8 ps Molecular Dynamics Simulation (MDS) was conducted for the SHV-1-avibactam and S130G-avibactam Michaelis-Menten and acyl-enzyme complexes as previously described and validated (19). A temperature of 300 K and a constant pressure were maintained during the heating/cooling, equilibration, and production stages of molecular dynamics simulation. The long-range electrostatics were treated with Particle Mesh Ewald and explicit solvation with Periodic Boundary Condition. These were run without any constraints.
The X-ray crystallography coordinates of CTX-M-15-avibactam were obtained (PDB ID: 4HBT). DS 3.1 was used to compare this X-ray crystallography structure with our acyl-enzyme model of SHV-1-avibactam.

Results and Discussion:

Microbiological activity of avibactam in combination with ampicillin against IR SHV variants expressed in E. coli DH10B

Table 2 summarizes the MICs that were determined for ampicillin and ampicillin-avibactam using a collection of IR SHV variants. Each group of variants (positions 69, 130, 234, 244, 276) is known to confer resistance to clavulanic acid by different mechanisms (4). Ceftazidime-avibactam was not reported as all variants were susceptible to ceftazidime (MIC ≤ 8 mg/L).

As shown in Table 2, avibactam is more effective at restoring the potency of ampicillin against the IR variants than other β-lactamase inhibitors (sulbactam, tazobactam, clavulanate). Avibactam lowers most of the MICs to ≤ 8 mg/L for ampicillin-avibactam. In these assays, 12/15 of the isolates have a MIC ≤ 8 mg/L for ampicillin-avibactam, 12/15 of the isolates have a MIC ≤ 128 mg/L for ampicillin-clavulanic acid, 12/15 of the isolates have a MIC ≤ 128 mg/L for piperacillin-tazobactam, and 12/15 of the isolates have a MIC ≥ 16384 mg/L for ampicillin alone. MICs were determined by maintaining the inhibitor concentration constant at 4 mg/L for clavulanic acid and sulbactam, while ampicillin concentration was increased and by holding a constant 8:1 ratio for piperacillin:tazobactam (this differs from previous work on these SHV variants where ampicillin was maintained at 50 mg/L and the inhibitor concentration was increased) (7, 11, 12, 14, 20). Despite these differences in MIC determinations, similar results are obtained showing a higher than expected MIC for the sulfone inhibitors even against SHV-1, which may be as a
result of the high enzyme expression level on the pBC SK (-) plasmid (20). The limitations of
clavulanic acid in inhibiting these enzymes is also highlighted in this previous work (7, 11, 12,
14, 20).

Interestingly, two variants of the SHV-1 β-lactamase stand out as showing elevated MICs
to ampicillin-avibactam and other BLI-β-lactam combinations, S130G and K234R. The
ampicillin MIC is only lowered one dilution after the addition of avibactam for the bacteria
containing the SHV S130G variant (from 512 mg/L to 256 mg/L, Table 3). The E. coli DH10B
expressing the SHV K234R variant also show an elevated ampicillin-avibactam MIC (MIC of
256 mg/L for ampicillin-avibactam vs. MIC of 16384 mg/L for ampicillin alone). This is
consistent with the observation that the K234R substitution was shown to affect the position of
the Ser-OH at Ambler position 130 (11). In contrast, all of the bacteria containing the SHV M69
variants show a lower MIC when avibactam is added to ampicillin (MICs < 8 mg/L), compared
to much higher MICs for the sulfone inhibitors (tazobactam and sulbactam) in combination with
piperacillin or ampicillin (MIC of 128-256 mg/L for piperacillin-tazobactam and MIC of 8192-
16384 for ampicillin-sulbactam).

As described above for S130G, the other S130 variants also provide interesting insights.
The S130A variant is severely impaired when expressed in E. coli DH10B (ampicillin MIC of 2
mg/L). The S130T variant in E. coli DH10B behaves similarly to SHV-1 in E. coli DH10B with
a fourfold lowering of the ampicillin MIC with the addition of avibactam. We maintain that since
threonine also has a hydroxyl group, this variant is similar to the wildtype serine at position 130
to allow avibactam to act as an inhibitor. As E. coli DH10B containing SHV S130G
demonstrated the least reduction in ampicillin MIC after the addition of avibactam, we decided
to further explore this variant enzyme to begin to understand the mechanistic basis for why it resists inhibition compared to the wildtype SHV-1.

Avibactam inhibition of the SHV β-lactamase

The inhibition of purified SHV-1 and SHV S130G with avibactam were studied in order to better understand the differences in inactivation of each of these enzymes. The results are summarized in Figure 2 and Table 3. Our analysis shows that the \( k_d/K \) value of the S130G variant of SHV-1 by avibactam is less than that of wildtype SHV-1 (1.3 M\(^{-1}\)s\(^{-1}\) vs 60,300 M\(^{-1}\)s\(^{-1}\), respectively). Additionally, we found that an approximately 17x higher avibactam concentration was required to achieve full SHV S130G inhibition compared to SHV-1 inhibition (Figure 2). In the context of these experiments, we interpret this to mean that the loss of the hydroxyl group at position 130 resulting from the Gly substitution impairs the initial productive contact between the β-lactamase and avibactam and formation of the acyl-enzyme bond between S70 and avibactam. Despite these differences, in a 24-hour time period the partition ratio \( (k_{cat}/k_{inac}) \) for both enzymes is identical at 1, indicating that avibactam inactivates both β-lactamases at a stoichiometric ratio over a long time period and is not hydrolyzed.

Mass-spectrometry of SHV β-lactamases

Figure 3 depicts the time course of SHV-1 or SHV S130G β-lactamase incubated with a 1:1 ratio of avibactam:enzyme during a time period between 1 min and 24 hr. As anticipated from our kinetic analysis, avibactam readily carbamylates SHV-1. Using the same approach, we observe that the formation of the acyl-enzyme species between avibactam and SHV S130G is much slower. As we examine the time course from 1 min incubation to 15 min incubation at a 1:1 ratio of inhibitor:enzyme, SHV S130G proceeds from minimally acylated enzyme to all acyl-enzyme. In contrast, the SHV-1 β-lactamase is fully acylated by avibactam at all time points. Importantly,
neither of these β-lactamases hydrolyzes avibactam as the acyl-enzyme complex between both β-
lactamases and avibactam continues to be observed at 1 hour and 24 hour time points. Additionally, on-enzyme fragmentation of avibactam is not observed for either β-lactamase during this time course as peaks of different masses were not observed. We interpret this to mean that avibactam does not undergo significant chemical rearrangements during the reaction and a highly stable adduct is formed between SHV and avibactam.

Molecular modeling of avibactam in SHV and S130G β-lactamases

Molecular modeling was performed to examine Michaelis-Menten and acyl-enzyme complexes of both SHV-1 and SHV S130G with avibactam in order to gain deeper insight into the mechanism of avibactam inhibition and resistance.

Initially, we compared our acyl-enzyme model of SHV-1-avibactam with the X-ray crystallographic structure of CTX-M-15-avibactam (PDB ID: 4HBT). The X-ray crystallography structure of the acyl-enzyme of CTX-M-15-avibactam shows that a proton shuttle occurs involving E166, a strategically placed water molecule, S70, avibactam, S130, and K73 (Figure 1B) (21). This mechanism of CTX-M-15 inhibition by avibactam was based on a high resolution structure showing deprotonation of E166 in the apo-CTX-M-15 structure and protonation of this residue after binding of avibactam (21). The comparison of the acyl-enzyme X-ray crystallography structure of CTX-M-15-avibactam with our acyl-enzyme model of SHV-1-avibactam (Figure 4) suggests that there may be a significant movement in the active site residues (i.e., S70, N132, E166, K234, and S/A237). This significant movement prompted us to propose that SHV-1 may be inhibited by avibactam by an alternative mechanism.

Which amino acid is the general base for avibactam acylation in the SHV-1 β-lactamase?
Comparing the SHV-1-avibactam acyl-enzyme model with the crystal structure of apo-SHV-1 (Figure 5A) showed there was repositioning among the active site amino acids (i.e., S130, E166, K234, and R244). Our MDS analysis of the SHV-1-avibactam acyl-enzyme complex further revealed that the catalytic water molecule that is critical for initiating acylation is absent. We propose that the carboxamide side chain of avibactam displaces this catalytic water molecule typically anchored by E166 and N170.

In the SHV-1:avibactam Michaelis-Menten complex, avibactam is within hydrogen bonding distance of S70, K73, E166, A237, and R244 and poised within the oxyanion hole in a favorable position for acylation. However, the catalytic water molecule is again absent (most likely due to displacement by the carboxamide of avibactam). Additionally, K73 is positioned more than 5 Å from S70 in all conformations in this model (Figure 6A). In contrast, S130 is within hydrogen bonding distance of S70. As the Lys73 and Glu166 acylation processes are not favored in this model (Figure 7A), we advance that S130 may also serve as the general base for acylation of the SHV β-lactamase by avibactam (Figure 7B). The CTX-M-15 X-ray crystallography structure support this hypothesis (note that due to the position of the carbamate bond of avibactam in the CTX-M-15-avibactam structure, E166, K73, or S130 may serve as the conjugate general base) (21). Furthermore, based on our model, we postulate that a proton shuttle occurs between K234, S130, and S70 for acylation of SHV-1 by avibactam (Figure 7B). Our other IR variants also support a different mechanism of inhibition of SHV-1 by avibactam compared to sulbactam, tazobactam, and clavulanic acid, as these variants (M69I/L/V, N276D) did not show elevated MICs to ampicillin-avibactam.

However, we note here, that the flexibility of the enzyme active site may allow water to enter, which would permit acylation to proceed through a traditional pathway involving E166.
The movement of bulk water transiently into the enzyme active site was previously postulated to occur in the R244S IR variant of SHV (14). Additionally, movement of K73 may also occur allowing it to serve as the general base for acylation.

Traditionally, acylation of the class A β-lactamases by β-lactams and β-lactamase inhibitors has been proposed to have two different pathways of initiation: 1) E166 or 2) K73 (4, 21-24). Quantum mechanical calculations have indicated that E166 is generally the most thermodynamically favored amino acid for initiation of acylation with the “K73 pathway” as an alternative means of initiating acylation (4, 23, 25). Evidence exists in the form of X-ray crystallography structures, thermodynamic calculations, nuclear magnetic spectroscopy, mutagenesis and kinetic measurements, and pH/pKₐ titration studies that support both E166 and K73 acting as the general base for the acylation of class A β-lactamases (21, 24, 26-31). There has also been molecular modeling and molecular dynamics simulation studies supporting the role of S130 in the acylation mechanism (31). There is no clear consensus of which pathway predominates as X-ray crystallography structures also conflict as to the protonation states of K73 and E166 and these protonation states may change in the transition from apo-enzyme, to Michaelis-Menten complex, to acyl-enzyme (21, 24, 27-29).

We are also aware that there are significant limitations to molecular modeling. Here, the timeframe for MDS was 8 ps and only one conformation (the most favorable) of avibactam was chosen for MDS. It is possible that with a longer MDS timeframe or a different initial avibactam conformation the active site water molecules would be observed. This would allow E166 to serve as the general base or alternatively that K73 would be within hydrogen binding distance of S70 to serve as the general base. We also note here that from our previous molecular modeling and compound docking, we have observed active site water molecules in enzyme-inhibitor...
complexes after similar modeling methodologies were used; avibactam appears to be the exception (12, 32). More extensive and sophisticated molecular modeling and molecular dynamics simulations in addition to high-resolution X-ray crystallographic structures, potentially NMR, and biochemical analysis of variants of SHV are required to determine the exact mechanism of avibactam inhibition of SHV. It is possible that competing acylation mechanisms may also exist.

Finally, we observe that upon acylation in SHV-1, avibactam is shifted such that S130 is within >3.5 Å of the sulfate amide proton (Figure 6C). S130 may also play a role in the recycrlization of avibactam from SHV-1 as proposed for CTX-M-15 (21). Thus, S130 may serve as the hydrogen acceptor to initiate recycrlization. Furthermore, given that the K234R variant also possess elevated ampicillin-avibactam MICs, K234 may play a role in proton shuttling to S130 allowing recycrlization to proceed unlike K73, which was proposed by Lahiri et. al. (21). Further experimentation will be required to differentiate these mechanistic details.

**Why is acylation impaired in the S130G variant of SHV?**

We compared our acyl-enzyme SHV S130G-avibactam model to the apo-SHV S130G crystal structure (PDB ID: 1TDL) and found that the active site amino acids were in similar locations between the two proteins (Figure 5B). The catalytic water molecule was also absent in the SHV S130 acyl-enzyme complex again due to steric interference from the carboxamide of avibactam.

The model of the SHV S130G:avibactam Michaelis-Menten complex disclosed that avibactam was uniquely positioned in the active site compared to SHV-1:avibactam Michaelis-Menten complex due to the loss of the S130 hydroxyl side chain (Figure 6B). As a result, there were fewer potential hydrogen bonding interactions between avibactam and the SHV S130G
variant enzyme in this model. However, we do observe that hydrogen bonding interactions were possible between K234 and avibactam and A237 and avibactam. As a Gly at position 130 does not have the hydroxyl group of Ser, this residue is unlikely to be involved in the acylation of the enzyme by avibactam as described above.

Previous X-ray crystallography of SHV S130G identified a water molecule in the active site of the enzyme compensating for loss of this hydroxyl group (8). In our model, we do not observe this water molecule during our MDS of the SHV S130G-avibactam representations (Figure 7C and Figure 5B). The reduced number of hydrogen bonds present in the Michaelis-Menten model between SHV S130G and avibactam may allow different conformations of the inhibitor to occur which permits two water molecules to enter the active site and allow acylation to proceed by a mechanism involving E166 as shown in Figure 7C. On the other hand, the distance between K73 and S70 is within hydrogen binding distance in the SHV S130G:avibactam molecular model (2.6 Å), which may lead K73 to initiate acylation with only one water molecule required to enter the active site to compensate for the missing hydroxyl group of S130 (Figure 7D). Again, we are limited by a lack of high-resolution crystallographic structures of SHV S130G revealing protonation states of K73 and E166. The same experiments required to identify the acylation (or competing acylation) mechanisms in SHV-1-avibactam are also needed to determine the major mechanism in SHV S130G. Overall, binding and acylation are less favorable in the S130G variant of SHV as the number of potential hydrogen bonding interactions between avibactam and the enzyme are decreased and the hydroxyl group of S130 is important in both potential acylation mechanisms of SHV-1 by avibactam.

An interaction with the sulfate amide proton of avibactam by the hydroxyl of S130 is also no longer possible as the Gly is now at position 130; thus recyclization is less favored (Figure
Additionally, in our models, the distance between the attacking nitrogen of avibactam and the carbonyl group bound to S70 is increased between the SHV-1-avibactam (3.4 Å) and the S130G-avibactam (3.7 Å), which suggests it is more difficult for avibactam to initiate the recyclization process in the S130G variant enzyme.

**S130 as a Final Common Pathway Underlying Inhibitor Resistance**

The location of S130 has been shown to be important in the IR phenotype not only of the S130G variant enzyme, but also of the K234R variant (11). The K234R substitution also impacts the ability of S130 to be involved in the proton shuttle with avibactam as this BLI is also ineffective at lowering the MIC for ampicillin against K234R (Table 3). Previous work advanced the hypothesis that the larger guanidinium sidechain of R234 leads to a movement of S130, which is then in an unfavorable position for acylation. In the case of avibactam, we believe that this movement of S130 in the K234R variant slows the proton shuttle allowing initiation of avibactam acylation. We also propose that K234 is involved in the proton shuttle for acylation and deacylation of SHV by avibactam and an Arg at this position would impair this proton movement.

Previous studies of N276D and R244S IR variant SHV enzymes proposed that the substitutions impaired the carboxylate recognition of inhibitors by these enzymes (12, 14). The SHV-1:avibactam Michaelis-Menten model reveals that R244 hydrogen bonds to the sulfate group of avibactam, which is analogous to the carboxylate recognition function of this amino acid for other substrates and inhibitors. As mentioned above, the N276D substitution does not show avibactam resistance as it did with clavulanic acid, which indicates that this amino acid does not serve the same role in recognition of avibactam. However, the observed avibactam resistance with the R244S substitution is likely due to the sulfate recognition by R244 that...
cannot be performed by a Ser residue. Alternatively, perhaps substitutions at this position affect
the location of the sulfate group of avibactam, which then leads to movement of S130. The close
spatial relationship between R244 and S130 in the SHV-1-avibactam molecular models (Figures
6A and 6B) supports a hypothesis involving interactions among these two amino acids.

Conclusions

The S130G substitution in the SHV class A β-lactamase, a natural variant, leads to
resistance to inhibition by avibactam, a novel non-β-lactam BLI. The mechanism of resistance
seems to be slow onset of acylation of the S130G enzyme by avibactam. There are several
possible amino acids that may initiate acylation of SHV and SHV S130G by avibactam
according to our molecular modeling (K73, S130, or E166). As the protonation states of these
residues are subject to debate, high resolution X-ray crystallographic structures and deeper and
more sophisticated molecular models and minimization are required to determine which
mechanisms are favored.

Since ceftazidime is effective against bacteria containing non-ESBL SHV enzymes, the
emergence of clinically significant avibactam-resistant isolates containing known IR
substitutions alone in the clinic is highly unlikely. However, we maintain that β-lactamases with
a combination of ESBL and IR substitutions of the SHV-type may evolve that exhibit resistance
to the ceftazidime-avibactam combination therapy as was observed in some CTX-M-9 class A β-
lactamase variants where an ESBL substitution restored hydrolysis of cephalosporins in a
sufficient quantity to allow an enzyme with the S130G substitution to display resistance to both
molecules (33). Additionally, as the S130G substitution seems to provide resistance to many
different classes of inhibitors and is present in SHV-10 which is found clinically (Table 1), it is
important to consider this substitution in addition to the K234R change which seems to be
intimately linked to the function of S130 and also a common clinical variant when developing future BLIs.

**Abbreviations**: Penicillin-binding protein (PBP), extended-spectrum β-lactamase (ESBL), β-lactamase inhibitor (BLI), minimum inhibitory concentration (MIC), clinical and laboratory standards institute (CLSI), European Committee on Antimicrobial Susceptibility Testing (EUCAST)

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**Acknowledgements**: We thank AstraZeneca Pharmaceuticals for providing the avibactam powder.

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15. CLSI. 2014. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. CLSI.


<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>β-lactamase</th>
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<tbody>
<tr>
<td>M69I</td>
<td>SHV-49, SHV-52, SHV-92</td>
</tr>
<tr>
<td>S130G</td>
<td>SHV-10</td>
</tr>
<tr>
<td>K234R</td>
<td>SHV-56, SHV-72, SHV-73, SHV-84</td>
</tr>
<tr>
<td>N276D</td>
<td>SHV-129</td>
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*aAdapted from (3)*
Table 2. MICs in mg/L of *E. coli* containing single IR SHV β-lactamases in the pBC SK (-) plasmid a, b

<table>
<thead>
<tr>
<th>Strain</th>
<th>AMP</th>
<th>AMP-AVI</th>
<th>AMP-SUL</th>
<th>AMP-CLAV</th>
<th>PIP</th>
<th>PIP-Tazo</th>
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<tbody>
<tr>
<td><em>E. coli</em> DH10B control</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
<td>1</td>
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<tr>
<td><em>E. coli</em> pBC SK (-) (\text{bla}_{\text{SHV-1}})</td>
<td>&gt;16384</td>
<td>1</td>
<td>&gt;16384</td>
<td>32</td>
<td>&gt;1024</td>
<td>256</td>
</tr>
<tr>
<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{M69I}})</td>
<td>8192</td>
<td>4</td>
<td>8192</td>
<td>128</td>
<td>1024</td>
<td>128</td>
</tr>
<tr>
<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{M69L}})</td>
<td>16384</td>
<td>8</td>
<td>16384</td>
<td>64</td>
<td>1024</td>
<td>256</td>
</tr>
<tr>
<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{M69V}})</td>
<td>16384</td>
<td>4</td>
<td>16384</td>
<td>128</td>
<td>1024</td>
<td>256</td>
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<tr>
<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{S130A}})</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>32</td>
<td>32</td>
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<tr>
<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{S130T}})</td>
<td>64</td>
<td>8</td>
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<td>0.5</td>
<td>256</td>
<td>128</td>
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<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{S130G}})</td>
<td>16384</td>
<td>8</td>
<td>8</td>
<td>8</td>
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<tr>
<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{N276A}})</td>
<td>512</td>
<td>256</td>
<td>256</td>
<td>254</td>
<td>1024</td>
<td>128</td>
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<tr>
<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{N276D}})</td>
<td>4096</td>
<td>2</td>
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<td>32</td>
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<td>64</td>
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<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{N276E}})</td>
<td>1024</td>
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<td>256</td>
<td>32</td>
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<td>0.5</td>
<td>256</td>
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<td>128</td>
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<td>16384</td>
<td>32</td>
<td>1024</td>
<td>128</td>
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<tr>
<td><em>E. coli</em> pBC SK (-) (\text{ble}_{\text{N276E}})</td>
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<td>1</td>
<td>2048</td>
<td>4</td>
<td>512</td>
<td>32</td>
</tr>
</tbody>
</table>

*a* *E. coli* DH10B purchased from Agilent, SHV-1 from (34), M69 variants from (13), S130 variants from (7), K234 variants from (11), R244 variants from (14), and N276 variants from (12), bAVI, SUL, CLAV held constant at 4 mg/L, TAZO kept at 8:1 ratio PIP:TAZO, cNaturally occurring variants.
Table 3. Inhibitory steady-state kinetic parameters for SHV-1 and the SHV-1 S130G variant with AVI.

<table>
<thead>
<tr>
<th></th>
<th>SHV-1</th>
<th>SHV-1 S130G</th>
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<tbody>
<tr>
<td>$K_m$ (μM)</td>
<td>12.5 ± 2.5</td>
<td>3107 ± 2393</td>
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<tr>
<td>$k_{cat}/K_m$ (s$^{-1}$μM$^{-1}$)</td>
<td>15.3</td>
<td>0.02</td>
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<tr>
<td>$k_2/K$ (M$^{-1}$s$^{-1}$)</td>
<td>60,300</td>
<td>1.3</td>
</tr>
<tr>
<td>$K_{i\text{app}}$ (μM)</td>
<td>0.022 ± 0.002</td>
<td>10274 ± 1000</td>
</tr>
<tr>
<td>24h partition ratio</td>
<td>1</td>
<td>1</td>
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Acylation of SHV-1 by Clavulanic Acid

Michaelis-Menten Complex

A

Acyl-Enzyme

Tetrahedral Intermediate

Figure 1A: Proposed acylation mechanism of SHV-1 by clavulanic acid (3, 5) 1B: Proposed acylation mechanism of CTX-M-15 by avibactam (20).
Figure 2A: Timed inactivation of SHV-1 by increasing concentrations of avibactam. The SHV S130G variant takes longer to plateau because of the lower NCF catalytic efficiency of this enzyme. Approximately a 1700x higher concentration of avibactam is required to inhibit the SHV S130G variant compared to SHV-1.
Figure 3: Mass spectrometry of SHV-1 and SHV S130G at various time points ranging from apo-enzyme to a 24 hr incubation of avibactam:enzyme at a 1:1 ratio.
Figure 4: Overlay of CTX-M-15-avibactam crystal structure (PDB ID: 4HBT, purple) with the acyl enzyme model of SHV-1-avibactam (blue) showing significant movement of many of the important active site residues and an absence of water molecules in the SHV-1-avibactam model.
Figure 5A: Overlay of apo-SHV-1 (PDB ID: 1SHV, purple) with our SHV-1-avibactam acyl-enzyme model showing significant movement of many of the active site residues in particular E166 and S130 and an absence of the bridging water molecule between these two residues in the acyl-enzyme model. Figure 5B: Overlay of apo-SHV S130G (PDB ID: 1TDL) with our SHV S130G-avibactam acyl-enzyme model showing similar locations of many of the residues but an absence of the two important water molecules in the SHV S130G-avibactam model.
Figure 6A: Michaelis-Menten complex model of SHV-1-avibactam 6B: SHV-1-avibactam acyl-enzyme model 6C: Michaelis-Menten complex model of SHV S130G-avibactam 6D: SHV S130G-avibactam acyl-enzyme model.
Figure 7: Proposed acylation mechanism of avibactam for the SHV β-lactamase (A and B) and the S130G variant enzyme (C and D).

Michaelis-Menten complex

Proposed tetrahedral intermediate

Acyl-enzyme

Acylation of S130G

No proton at position 130 for shuttle

Very slow acylation

Acyl-enzyme

Alternative Acylation of S130G

Michaelis-Menten complex

Proposed tetrahedral intermediate

Acyl-enzyme

Alternative Acylation of SHV-1

Michaelis-Menten complex

Proposed tetrahedral intermediate

Acyl-enzyme

Acylation of SHV-1

Michaelis-Menten complex

Proposed tetrahedral intermediate

Acyl-enzyme

2.6Å

transient water?

transient water?

transient water?