Overcoming Resistance to β-Lactamase Inhibitors: Comparing Sulbactam to Novel Inhibitors against Clavulanate Resistant SHV Enzymes with Substitutions at Ambler Position 244†

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ABSTRACT: Amino acid changes at Ambler position R244 in class A TEM and SHV β-lactamases confer resistance to ampicillin/clavulanate, a β-lactam/β-lactamase inhibitor combination used to treat serious infections. To gain a deeper understanding of this resistance phenotype, we investigated the activities of sulbactam and two novel penem β-lactamase inhibitors with sp^2 hybridized C_3 carboxylates and bicyclic R1 side chains against a library of SHV β-lactamase variants at the 244 position. Compared to SHV-1 expressed in *Escherichia coli*, all 19 R244 variants exhibited increased susceptibility to ampicillin/sulbactam, an important difference compared to ampicillin/clavulanate. Kinetic analyses of SHV-1 and three SHV R244 (-S, -Q, and -L) variants revealed the K_i for sulbactam was significantly elevated for the R244 variants, but the partition ratios, k_{cat}/k_{inact}, were markedly reduced (13 000 → ≤500). A timed inactivation−mass spectrometry analysis of SHV inhibited by sulbactam showed that SHV-1 β-lactamase was unmodified at 15 min. A parallel experiment with R244S demonstrated 70 and 88 ± 3 Da fragments of sulbactam covalently attached to the β-lactamase. We also observed that the K_i values of penems **1** and **2** were increased for R244 variants compared to those for SHV; however, these inhibitors effectively restored ampicillin susceptibility in vitro. Compared to that of sulbactam, the k_{cat}/k_{inact} values of penems for SHV-1 and R244S were low (≤2), and unfragmented adducts of each penem were covalently attached to SHV-1 and R244S when studied using the timed inactivation−mass spectrometry method. The R244S mutation affects β-lactamase inactivators differently, but resistance can be overcome by designing penem inhibitors with strategic chemical properties that improve affinity and impair turnover.

β-Lactam antibiotics (e.g., penicillins, cephalosporins, and carbapenems) are bactericidal agents that target penicillin binding proteins (PBPs),[1] the essential enzymes in cell wall biogenesis. The use of β-lactams in the clinic is constantly threatened by the ever-expanding numbers of β-lactamases, now numbering more than 700 unique enzymes (EC 3.5.2.6). Divided into four classes (A−D) based on amino acid homology, β-lactamases demonstrate a diverse substrate specificity (*1*). The most commonly encountered β-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* (TEM and SHV, respectively) are class A enzymes (*2*, *3*). Single-amino acid substitutions in the TEM and SHV β-lactamases are responsible for the current shortage of penicillins and cephalosporins active against β-lactamase-mediated resistance in common enteric bacilli (*1*, *2*, *4*).

β-Lactamase inhibitors preserve the clinical utility of penicillins by inactivating β-lactamases and allowing the β-lactam to reach the PBPs. Three β-lactamase inhibitors are commercially available: clavulanate, tazobactam, and sulbactam (Figure 1). The β-lactam/β-lactamase inhibitor formulations (amoxicillin/clavulanate, ticarcillin/clavulanate, ampicillin/sulbactam, cefoperazone/sulbactam, and piperacillin/tazobactam) are widely used to treat complicated infections due to β-lactam resistant organisms. Unfortunately, resistance to β-lactam/β-lactamase inhibitor combinations is a growing clinical problem that is increasingly important among Gram-negative pathogens harboring class A β-lactamases. Several inhibitor resistant TEM (IRT) variants have arisen with substitutions at Ambler position R244 (R244S, -L, -C, -T, -H, and -G) (*1*). Inhibitor resistant variants at Ambler position M69 and S130 are also described in the TEM and SHV family of β-lactamases (*5*−*7*). Detailed kinetic and structural studies that explain the resistance observed to clavulanate among IRTs (*8*−*12*) and inhibitor resistant SHV β-lactamases (*13*−*16*) are being performed.

Anticipating that inhibitor resistance phenotypes in SHV β-lactamases will arise with similar alterations as IRTs, we initially studied the effects of substitutions at R244 in SHV

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[1] Abbreviations: PBP, penicillin binding protein; IRT, inhibitor resistant TEM; MIC, minimal inhibitory concentration; ESI-MS, electrospray ionization mass spectrometry; WT, wild-type SHV-1.

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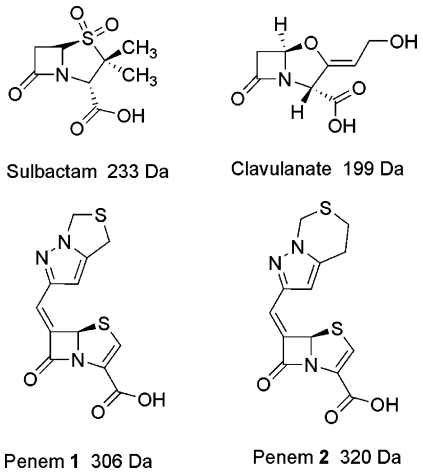

FIGURE 1: Chemical structures of the commercially available β-lactamase inhibitors, sulbactam, and clavulanic acid, as well as the novel inhibitors, penems **1** and **2**.

β-lactamases and showed that 15 variants at R244 exhibited increased resistance to clavulanate (*16*). We also observed that the kinetic basis of the clavulanate resistant phenotype was a decreased affinity of the enzymes for clavulanate (60−1000-fold increases in K_i), presumably through the loss of bonding to the C_3 carboxylate. Interestingly, we also detected an increase in the rates of inactivation (k_{inact}) and, in some cases, increases in the partition ratio (k_{cat}/k_{inact}) among clavulanate resistant mutants.

To gain deeper insight into the basis of resistance to β-lactamase inhibitors among class A enzymes that have substitutions at Ambler position R244, we studied the phenotypic properties and steady state kinetic interactions of a commercially available sulfone inhibitor, sulbactam (Figure 1), against the wild type (WT) and three clavulanic acid resistant variants of SHV-1 with substitutions at the 244 position (R244S, R244Q, and R244L). Since our analysis of the turnover of Meropenem with SHV-1 and R244 variants highlighted the importance of the interaction of R244 with the C_3 carboxylate, we were also compelled us to examine investigational penem-type β-lactamase inactivators (penems **1** and **2**) against these clavulanate resistant SHV-type β-lactamases (*16*). Penems **1** and **2** were chosen because they possess an sp^2 hybridized carboxylate at the C_3 position and a bicyclic R_1 side chain and differ from clavulanate, sulbactam, and tazobactam in their mechanism of inactivation of class A β-lactamases (*17*−*19*).

Our studies reveal that sulbactam is a poor inhibitor of WT because of an extremely high partition ratio, as also documented for the TEM-1 β-lactamase (*20*). Substitutions at R244 in SHV reverse this phenotype, restoring some susceptibility, but not to clinically effective levels. We also show that penems **1** and **2** are able to lower the minimum inhibitory concentrations, MICs, of ampicillin against WT and R244 mutants of SHV-1. By elucidating the interactions of these mechanism-based β-lactamase inactivators against WT and inhibitor resistant variants of SHV β-lactamase at the 244 position, we obtained important insights into novel drug design.

MATERIALS AND METHODS

Plasmids and Mutagenesis. bla_{SHV-1}, subcloned into phagemid vector pBC SK(−) (Stratagene, La Jolla, CA) and maintained in ElectroMAX DH10B $T1^R$ cells (Invitrogen, Carlsbad, CA) (*21*), served as the template for all mutageneses at Ambler position R244 described previously (*16*).

Antibiotic Susceptibility. Minimal inhibitory concentrations (MICs) were determined by the agar dilution method, using a Steer's Replicator that delivers 10^4 cfu per 10 μL spot. Susceptibilities of sulbactam (Pfizer, La Jolla, CA) and penems **1** and **2** (Wyeth Pharmaceuticals, Madison, NJ) were determined by increasing the concentration of inhibitor in the presence of 50 μg/mL ampicillin. Reported values are the most frequent MIC observed (mode) in at least three separate experiments.

Protein Expression and Purification. E. coli DH10B cells containing the bla_{SHV-1} and $bla_{SHV\ R244S,\ -Q,\ and\ -L}$ genes in pBC SK(−) were grown overnight in SOB medium, harvested by centrifugation at 4 °C, and frozen. β-Lactamase was liberated using stringent periplasmic fractionation with 40 μg/mL lysozyme and 1 mM EDTA (pH 7.8). Preparative isoelectric focusing was performed with the lysate in a Sephadex granulated gel using ampholines in the pH range of 3.5−10 (Amersham Biosciences) as previously described (*22*). The protein was eluted and dialyzed with 20 mM diethanolamine buffer (pH 8.5). The protein concentration was assessed with a Bio-Rad (Hercules, CA) protein assay with bovine serum albumin standards. The purity of >95% was determined using 10% SDS−PAGE.

Kinetics. Kinetic constants of the β-lactamases chosen for the study (SHV-1 and R244S, -Q, and -L) were measured by continuous assays at room temperature (25 °C) using an Agilent (Palo Alto, CA) 8453 diode array spectrophotometer. The kinetic parameters were obtained with a nonlinear least-squares fit of the data using Origin 7.5. Detailed descriptions of the assays performed and data analysis were previously published (*16*).

Mass Spectrometry. For intact protein mass spectrometry, 40 μM SHV-1 or R244S was incubated for 15 min with and without the addition of 40 mM sodium sulbactam, or 20 mM penem **1** or **2**. Each sulbactam reaction was terminated by the addition of 1/10 volume of 1% trifluoroacetic acid, and the mixture was immediately desalted and concentrated using a C_4 ZipTip (Millipore, Bedford, MA) according to the manufacturer's protocol. Reactions with penems were not terminated with the addition of acid, as this precipitated the samples. These were desalted "as is" using a C_4 ZipTip. Samples were then placed on ice and analyzed within 10 min.

Spectra of the intact SHV-1 and SHV R244S proteins were generated on an Applied Biosystems (Framingham, MA) Q-STAR XL quadrupole time-of-flight mass spectrometer equipped with a nanospray source. Experiments were performed by diluting the protein sample with acetonitrile and 1% formic acid to a concentration of 10 μM. This protein solution was then infused at a rate of 0.5 μL/min, and data were collected for 2 min. Spectra were deconvoluted using Analyst from Applied Biosystems.

Structure Analysis. Protein Data Bank coordinates of SHV were examined using ViewerLite (Accelrys, San Diego, CA). The following Research Collaboratory for Structural Bioinformatics Protein Data Bank entries were analyzed: 2A3U (SHV E166A with sulbactam) (*23*) and 2A49 (SHV E166A with clavulanate) (*23*).

Table 1: MICs (micrograms per milliliter) of *E. coli* DH10B Containing SHV-1 and All 19 Variants at Arg244[a]

	ampicillin[c]	clavulanate[a,c]	sulbactam[a]	penem **1**[a]	penem **2**[a]
Arg	**>16384**	**2**	**256**	**0.25**	**2**
Ala	256	4	32	1	8
Asn	256	4	32	0.5	8
Asp	2	N/A[d]	N/A[d]	N/A[d]	N/A[d]
Cys[b]	128	2	32	0.5	8
Gln	**1024**	**16**	**64**	**1**	**16**
Glu	256	8	32	1	8
Gly[b]	256	4	32	0.5	8
His[b]	1024	8	64	2	16
Ile	256	8	16	1	16
Leu[b]	**128**	**16**	**32**	**1**	**16**
Lys	2048	8	128	0.25	4
Met	512	16	64	1	16
Phe	256	8−16	32	1	16
Pro	1	N/A[d]	N/A[d]	N/A[d]	N/A[d]
Ser[b]	**1024**	**8**	**32−64**	**2**	**16**
Thr[b]	512	8	32	1	16
Trp	8	N/A[d]	N/A[d]	N/A[d]	N/A[d]
Tyr	128	16	16	1	8
Val	128	8−16	32	1	16

[a] Inhibitors were evaluated in the presence of 50 μg/mL ampicillin. Bold type denotes variants chosen for kinetic analysis. [b] Substitutions found clinically in TEM. [c] Previously published (*16*). [d] Mutants had a MIC of <50 μg/mL ampicillin and so could not be tested.

RESULTS

Antibiotic Susceptibility. To assess the impact of each amino acid substitution on the in vivo kinetic behavior of SHV β-lactamase, we first determined MICs. Listed in Table 1 are the results of our susceptibility testing. *E. coli* DH10B cells containing bla_{SHV-1} encoded in the pBC SK(−) phagemid vector exhibit robust levels of resistance to ampicillin (>16000 μg/mL). High-level resistance to ampicillin/sulbactam was also demonstrated (50 μg/mL ampicillin and 256 μg/mL sulbactam). In contrast, 18 of 19 SHV-1 variants with substitutions at position 244 expressed in this uniform genetic background were more susceptible to ampicillin and sulbactam than SHV-1 (Table 1). Only *E. coli* DH10B with R244K β-lactamase (MIC = 50 μg/mL ampicillin/128 μg/mL sulbactam) maintained resistance comparable to that of WT (MIC = 50 μg/mL ampicillin/256 μg/mL sulbactam). In general, the reduction in resistance to the ampicillin/sulbactam combination was 2−4-fold. The most sulbactam susceptible mutants demonstrated a sulbactam MIC of 16 μg/mL when combined with ampicillin, 50 μg/mL. This pattern of increased susceptibility to a β-lactamase inhibitor is in sharp contrast to what was observed with ampicillin/clavulanate (*16*).

Compared to clavulanate and sulbactam, penem **1** is an extremely potent inhibitor of SHV-1. Penem **1** at 0.25 μg/mL lowered resistance to ampicillin in *E. coli* DH10B cells expressing SHV-1 (Table 1). Although all variants at position 244 were shown to be more resistant to penem **1** than WT expressed in *E. coli* (the pattern seen for clavulanate), the level of resistance remained much lower than that for ampicillin/sulbactam (MICs ranged from 0.06 to only 2 μg/mL). The highest degree of resistance was seen with the R244H and R244S variants (50 μg/mL ampicillin/2 μg/mL penem **1**). Penem **2** combined with ampicillin was also more effective than sulbactam with ampicillin but was less potent than penem **1** against the R244 mutants. Fifteen of 20 variant β-lactamases with substitutions at R244 had elevated MIC values (≥50 μg/mL ampicillin/8 μg/mL penem **2**) compared to a MIC of WT (50 μg/mL ampicillin/2 μg/mL penem **2**) (Table 1).

*Kinetic Behavior of Clavulanate Resistant β-Lactamases with Sulbactam and Penems **1** and **2***. Because of their clinical importance and high ampicillin/clavulanate MICs, we chose R244S, -Q, and -L for further kinetic characterization (*24*, *25*). In light of our susceptibility testing, finding an elevated sulbactam K_i against these three variants was unanticipated (Table 2). In competition reactions with nitrocefin, we observed that the dissociation constants for the preacylaction complexes were increased 28−140-fold, despite the MIC result that all variants were shown to be more susceptible to sulbactam.

Substitutions at Ambler position 244 also drastically altered the enzyme's ability to hydrolyze sulbactam. SHV-1 exhibited a very high partition ratio for this sulfone (k_{cat}/k_{inact} = 13 000 ± 100) with k_{cat} = 730 ± 4 s^{-1}. In comparison, all three R244 clavulanic acid resistant β-lactamases exhibited partition ratios of ≤500, the lowest being that of the R244S enzyme (k_{cat}/k_{inact} = 100). After accounting for increased k_{inact} values among the mutants, we observed that the k_{cat} values for sulbactam were decreased 10−55-fold. This reduction in catalytic efficiency contributes to the increase in susceptibility.

The K_i values of penems **1** and **2** were in the nanomolar range for WT SHV-1 β-lactamase (14 ± 2 and 48 ± 4 nM, respectively) (Table 3). In parallel with our observations with sulbactam, we again observed a loss of affinity among the mutants at position R244. There was an 80−280-fold (14 nM → 3.9 μM) and 90−260-fold (48 nM → 12.5 μM) increase in K_i for penem **1** and **2**, respectively. The most striking feature of the penem inhibitors, however, is an extremely low rate of turnover when tested against the selected enzymes. Both penems **1** and **2** exhibited partition ratios (k_{cat}/k_{inact}) of 2 for SHV-1 and 1 for R244S, -Q, and -L (Table 3). The resulting k_{cat} values (<0.4 s^{-1}) are lower than those seen for clavulanate or sulbactam.

Mass Spectrometry of SHV-1 and R244S with Sulbactam and the Penem Inhibitors. To discern the nature of the intermediates of inactivation by sulbactam and the penem inhibitors in the reaction pathway of SHV-1 and R244S, we performed electrospray ionization mass spectrometry (ESI-MS). When sulbactam was being studied, a 1000:1 molar ratio of this inhibitor, I, to enzyme, E, was prepared, and the reaction proceeded for 15 min before being terminated with trifluoroacetic acid. In the case of SHV-1, we observed a predominance of unmodified β-lactamase at the predicted molecular mass of 28 872 ± 3 Da (Figure 2). Minor peaks include adducts of Δ + 71 ± 3, Δ + 90 ± 3, Δ + 115 ± 3, Δ + 142 ± 3, and Δ + 228 ± 3, most of which have been previously documented (*5*, *14*, *26*). A markedly different result is seen when the inactivation of R244S is studied. Using an I:E ratio of 1000:1, the predominant intermediates are the Δ + 70 ± 3 and Δ + 88 ± 3 Da adducts, with minor peaks representing adducts of Δ + 105 ± 3, Δ + 131 ± 3, and Δ + 202 ± 3 Da. We did not detect unmodified R244S β-lactamase (28 803 ± 3 Da) after inactivation with sulbactam for 15 min.

Because of the greater affinity and lower partition ratios of the penem inhibitors for SHV-1, we inactivated WT and R244S using a 500:1 I:E ratio. Unlike our observations with

Table 2: Kinetic Properties of SHV-1 β-Lactamase and Variants at Ambler Position R244 for Sulbactam and Clavulanate[a]

	SHV-1	R244S	R244Q	R244L
		Sulbactam		
K_i (μM)	8.6 ± 0.7	240 ± 20	510 ± 34	1200 ± 100
k_{inact} (s^{-1})	0.056 ± 0.003	0.13 ± 0.01	0.14 ± 0.01	0.19 ± 0.03
k_{inact}/K_i (μM^{-1} s^{-1})	0.0065 ± 0.0006	0.00054 ± 0.00006	0.00027 ± 0.00003	0.00016 ± 0.00003
k_{cat}/k_{inact}	13000 ± 100	100 ± 10	500 ± 20	300 ± 20
k_{cat} (s^{-1})	730 ± 40	13 ± 2	70 ± 6	57 ± 10
		Clavulanate[a]		
K_i (μM)	1 ± 0.04	63 ± 3	85 ± 7	360 ± 20
k_{inact} (s^{-1})	0.04 ± 0.002	0.09 ± 0.005	0.09 ± 0.007	0.11 ± 0.01
k_{inact}/K_i (μM^{-1} s^{-1})	0.04 ± 0.003	0.0014 ± 0.0001	0.0011 ± 0.0001	0.00031 ± 0.00003
k_{cat}/k_{inact}	60 ± 10	50 ± 10	180 ± 10	550 ± 30
k_{cat} (s^{-1})	2.4 ± 0.4	4.5 ± 0.9	19 ± 2	61 ± 6

[a] See also ref *16*.

Table 3: Kinetic Properties of SHV-1 β-Lactamase and Variants at Ambler Position R244 for Penem **1** and Penem **2**

	SHV-1	R244S	R244Q	R244L
		Penem **1**		
K_i (μM)	0.014 ± 0.002	1.1 ± 0.1	1.2 ± 0.1	3.9 ± 0.4
k_{inact} (s^{-1})	0.17 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.02
k_{inact}/K_i (μM^{-1} s^{-1})	12.3 ± 1.6	0.14 ± 0.02	0.12 ± 0.01	0.038 ± 0.007
k_{cat}/k_{inact}	2 ± 1	1 ± 1	1 ± 1	1 ± 1
k_{cat} (s^{-1})	0.35 ± 0.17	0.15 ± 0.07	0.14 ± 0.07	0.15 ± 0.08
		Penem **2**		
K_i (nM)	0.048 ± 0.004	4.4 ± 0.6	4.6 ± 0.3	12.5 ± 0.7
k_{inact} (s^{-1})	0.186 ± 0.008	0.159 ± 0.007	0.140 ± 0.009	0.116 ± 0.007
k_{inact}/K_i (μM^{-1} s^{-1})	3.9 ± 0.3	0.037 ± 0.002	0.031 ± 0.003	0.0093 ± 0.0008
k_{cat}/k_{inact}	2 ± 1	1 ± 0.5	1 ± 0.5	1 ± 0.5
k_{cat} (s^{-1})	0.37 ± 0.19	0.16 ± 0.08	0.14 ± 0.07	0.12 ± 0.06

sulbactam and clavulanate, we did not see evidence of inhibitor fragmentation with the penems (Figure 3). For both penems **1** and **2** with SHV-1 and R244S, we observed adducts of $\Delta + 307 \pm 3$ for penem **1** and $\Delta + 321 \pm 3$ for penem **2**. In addition, uninhibited enzyme is not present after incubation with either β-lactamase for 15 min. Curiously, there appear to be smaller peaks that correspond to multiple penem adducts for each enzyme (Figure 3).

DISCUSSION

The paradoxical increased susceptibility to ampicillin/sulbactam in this panel of ampicillin/clavulanate resistant isolates containing SHV β-lactamases with substitutions at R244 is intriguing. Eighteen of 19 substitutions at R244 lead to β-lactamases with increased susceptibility to ampicillin/sulbactam by two or more dilutions. Equally surprising were our results with penem **1** and, to a lesser extent, penem **2**; the analysis of Meropenem inactivation of R244S, -Q, and -L variants of SHV predicted that penem inhibitors may not be effective because of the position of the sp^2 hybridized carboxylate at the C_3 position (K_i values were increased 1000-fold).

To understand the mechanistic basis of this phenotypic behavior, we compared the dissociation constants for the preacylation complexes of SHV-1, R244S, R244Q, and R244L β-lactamases against each inhibitor. Our data show that the principal consequence of R244 substitutions in SHV-type β-lactamases that were studied is an increase in K_i values for sulbactam (*16*, *20*, *27*). This observation is also common among IRT β-lactamases as well as other SHV variants (*5*, *28*, *29*). To analyze the inactivation process further, we next examined the partition ratio. Of central importance is our finding that sulbactam, a sulfone, is readily hydrolyzed by SHV-1 ($k_{cat} = 730 \pm 40$ s^{-1}). This k_{cat} may be the biochemical basis for the elevated MIC values (*30*). By substituting Ser for Arg at position 244, we see a 56-fold reduction in k_{cat} for sulbactam; this decrease is accompanied by a more efficient inactivation of the β-lactamase (a 10-fold difference in k_{inact}/K_i). We emphasize that although R244 substitutions decrease the rate of hydrolysis of sulbactam, the opposite is seen for clavulanate; R244S, R244Q, and R244L hydrolyze clavulanate from 1.9- to 25-fold faster than WT. It is remarkable that the same substitutions can have such opposite effects on the turnover of these two inhibitors (*26*).

We compared the data obtained in this study with similar analyses performed that examined the inactivation of the TEM R244S, -C, and -H (TEM-30, -31, and -51, respectively) β-lactamases with sulbactam (*20*, *27*). Although these investigations also elucidated elevated K_i values, it is noteworthy that the R244 substitutions in TEM lead to β-lactamases with >3-fold reductions in their sulbactam k_{inact} values. This markedly contrasts with our data with SHV, which demonstrate a >2-fold increase in k_{inact} for sulbactam for the variants that were studied. These considerations, combined with our previous kinetic data for clavulanate, indicate that the "enzymatic machinery" of these two class A β-lactamases is distinctly different vis-à-vis inhibitors.

These kinetic observations are supported by timed mass spectrometry studies that mapped the inactivation of WT and R244S by sulbactam. After a 15 min inactivation, the mass spectroscopic analysis of the 1000:1 sulbactam/SHV-1 mixture revealed a large, dominant peak at 28 872 ± 3 Da; multiple small peaks suggesting adducts at 70 ± 3, 90 ± 3,

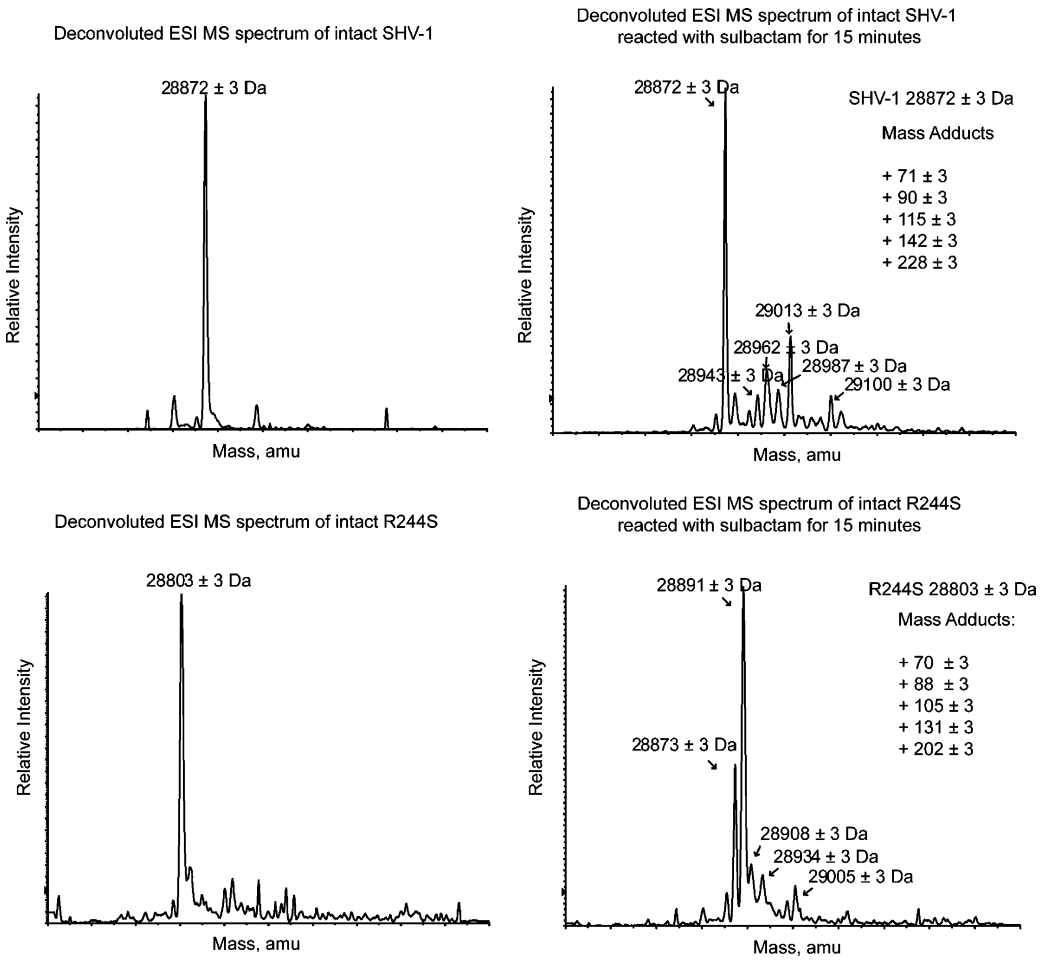

FIGURE 2: Deconvoluted ESI-MS spectra of SHV-1 (WT) and R244S β-lactamase reacted with a 1000:1 sulbactam/enzyme mixture for 15 min. Notice that at this time point most of the SHV-1 is unmodified while all of the R244S has multiple adducts.

115 ± 3, 142 ± 3, and 228 ± 3 Da are also noted. In contrast, when the R244S variant was inactivated, we found primarily 70 ± 3 Da (28 873 ± 3 Da) and 88 ± 3 Da adducts (28 891 ± 3 Da) which we assign to the aldehyde and semialdehyde intermediates, respectively. Of note, similar products were seen in the inactivation of S130G with tazobactam and clavulanate (*5*).

Clues about the basis of this dynamic difference in inhibitor turnover in SHV can be gleaned from examining the atomic structures of the SHV E166A β-lactamase with the *trans*-enamine intermediates of clavulanate (Figure 4A) and sulbactam (Figure 4B) trapped in the active site (*23*, *31*). Interpretation of these two structures reveals that R244 contributes to the structural stability of the β-sheet "network" that comprises the "right side" of the binding site pocket. This is accomplished through hydrogen bonding interactions of the R244 side chain with the side chain of N276 and the backbone amide of G236, as well as hydrogen bonding interactions between the backbones of R244 and L265 (Figure 4). Removing these stabilizing contacts in R244S is predicted to enlarge the active site pocket. We also note that sulbactam is oriented differently in the active site pocket compared to clavulanate; clavulanate is slanting toward the B3 β-sheet network, while sulbactam is positioned straight out of the active site pocket (Figure 4). Disruptions in the β-sheet network, which coordinate a water molecule that may be repositioned into the active site with the binding of either clavulanate or sulbactam, are more likely to have an adverse effect on sulbactam hydrolysis by increasing critical hydrogen bond distances. Even though the resulting MICs against ampicillin/sulbactam when R244 is substituted are lower, the level of resistance remains too high for the combination to regain therapeutic efficacy.

On the basis of our studies of Meropenem inactivation of SHV-1 and R244S, we next explored the interactions of novel inhibitors with sp^2 hybridized carboxylates at the C_3 position. We observed that the dissociation constants for the preacylation complexes of penems **1** and **2** for inhibitor resistant SHV β-lactamases with substitutions at R244 are in the low micromolar range ($K_{i\ WT} = 0.014 \pm 0.002 \rightarrow K_{i\ R244S} = 1.1 \pm 0.1\ \mu M$). However, there is no significant change in the k_{cat} or partition ratios. As a result, penems **1** and **2** were able to restore the susceptibility of ampicillin against *E. coli* containing the inhibitor resistant SHV β-lactamases.

Evidence of inhibitor fragmentation or deacylation is lacking when mass spectrometry studies are performed; this finding is consistent with our kinetic analysis that indicated a remarkably low rate of inhibitor turnover. In this analysis (Figure 3), we observe that only the 307 ± 3 and 321 ± 3 Da adducts are formed. We also suspect that there is nonspecific (or non-active site) binding of a second inhibitor molecule to the WT and R244S β-lactamase. In each of the 15 min deconvoluted spectra, we observe a minor 615 ± 3 and 643 ± 3 Da adduct to the β-lactamase enzymes.

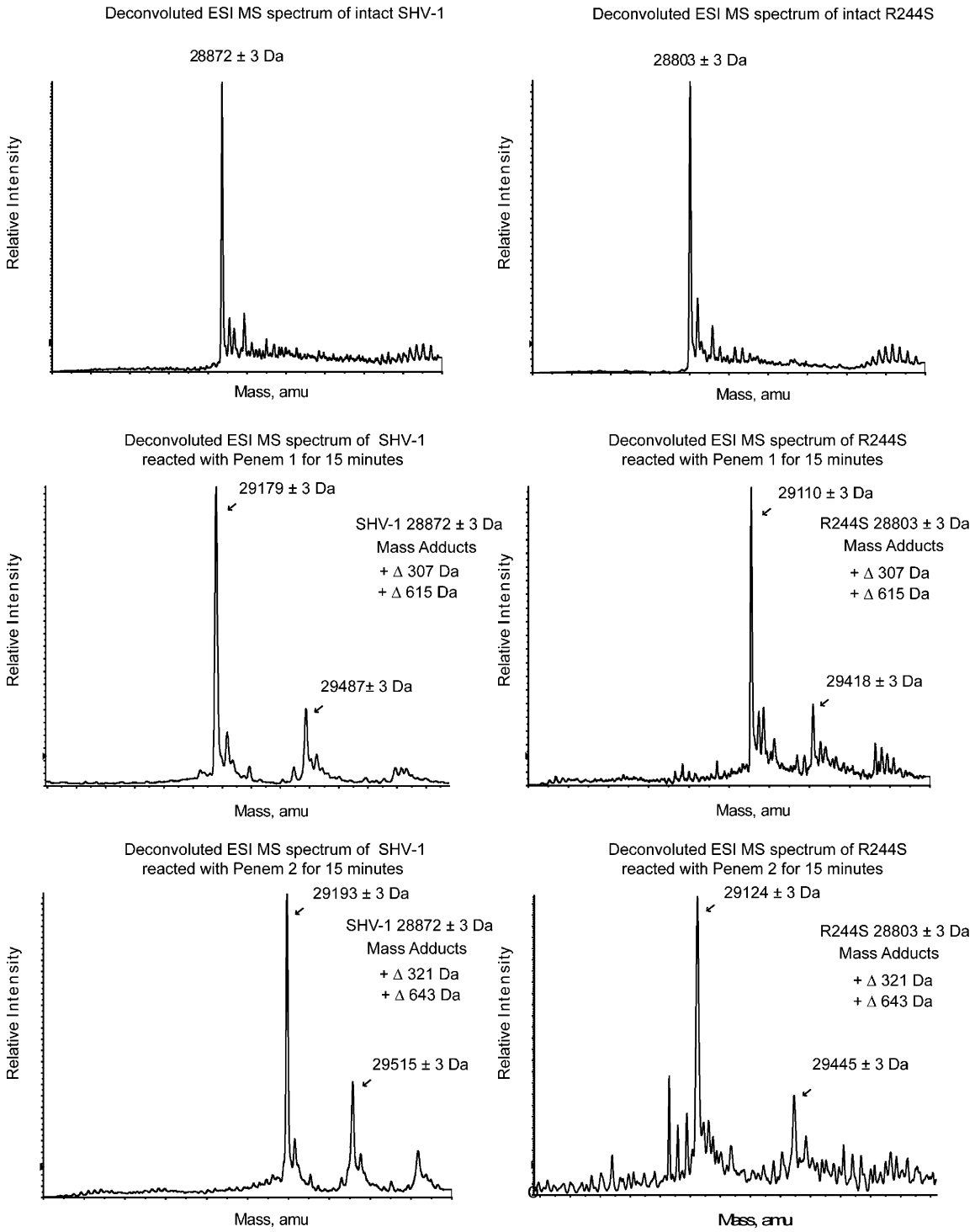

FIGURE 3: Deconvoluted ESI-MS spectra of SHV-1 and R244S β-lactamase reacted with 500:1 penem **1**/enzyme and 500:1 penem **2**/enzyme mixtures for 15 min. At this time point, unreacted SHV-1 or R244S is not observed. The mass spectrometry analysis of the reaction indicates that the inhibitor covalently attaches to the enzyme without fragmentation, which is seen with sulbactam. Interestingly, there is some evidence of the addition of multiple inhibitor molecules. As stated in the text, we interpret this as representing nonspecific addition.

To explain these results, we advance that two important properties of these investigational penems are responsible for their low K_i and partition ratios. First, the bicyclic R1 side chain may compensate for the loss of the interactions of the C_3 carboxylate with R244. Using previous atomic structure determinations and molecular modeling studies as a foundation for this statement, we propose that favorable $\pi-\pi$ interactions with the aromatic side chain of Y105 and the R1 side chain occur (*18*). To contrast, clavulanate and sulbactam lack R1 side chains and are highly dependent in the H bonding interactions of the C_3 carboxylate (K_i increases from 1 to >1000 μM). Second, penem inhibitors **1** and **2** follow a different path to inactivation in serine β-lactamases (*17*, *32*). Unlike clavulanate and sulbactam which undergo secondary ring opening, formation of the linear imine and *cis*,*trans*-enamine on their way to the final products of inactivation, penems **1** and **2** acylate the β-lactamase and likely undergo *endo* trig cyclization. This results in the appearance of a seven-membered cyclic adduct (Figure 5) (*18*). The rapid formation of a seven-membered cyclic

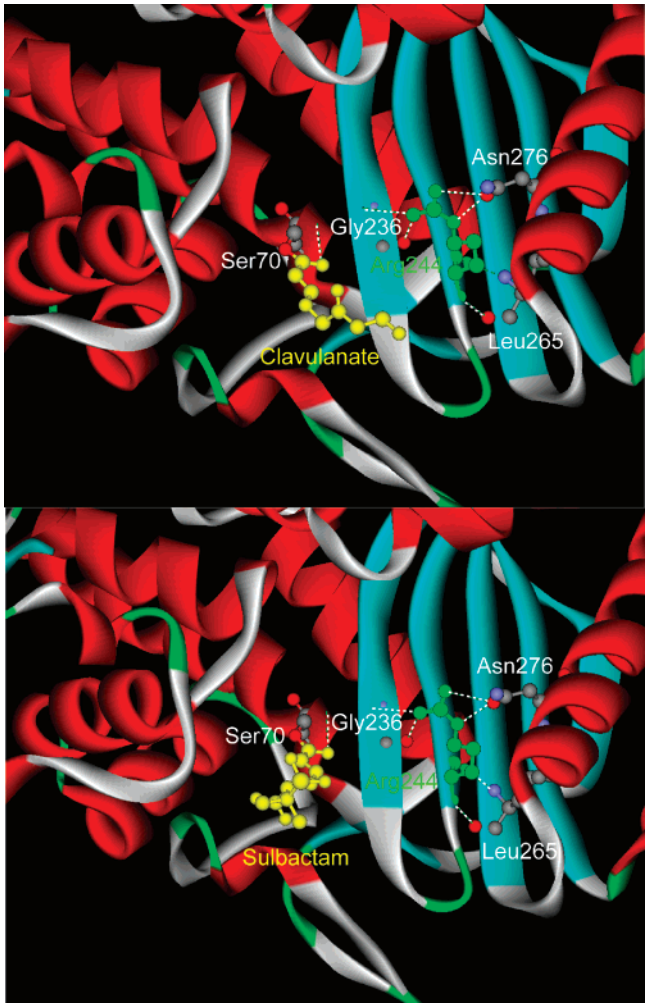

FIGURE 4: Molecular representation of SHV E166A with the *trans*-enamine intermediate of clavulanate (top) and sulbactam (bottom) (based upon PDB coordinates 2A49 and 2A3U, respectively). Important residues for interactions with R244 (green) are shown, and the inhibitors are highlighted in yellow.

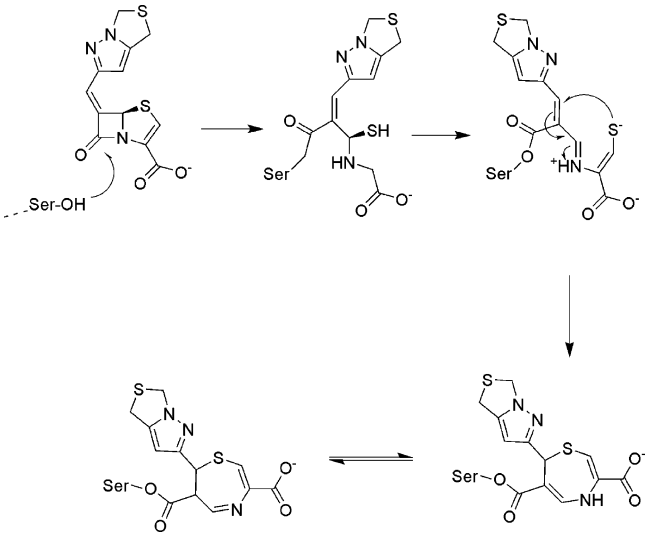

FIGURE 5: Proposed mechanism of inactivation of penem **1** with WT and R244S β-lactamase. Here, we illustrate that *endo* trig cyclization of penem **1** with SHV-1. This pathway results in an enzyme adduct equal to the size of the unfragmented inhibitor molecule.

enamine within the active site may also displace the catalytic water molecule and reduce the rate of turnover (*17*, *18*).

In conclusion, the main challenges in β-lactamase inhibitor development are (i) discovering why single-amino acid changes alter inhibitor affinity and turnover and (ii) developing novel inhibitors with activity against a broad spectrum of inhibitor susceptible and resistant enzymes. Future advances in inhibitor design against these versatile β-lactamases must incorporate strategies that address each of the key structural features of these diverse proteins as well steps in enzymatic inactivation. Considered together (high affinity, formation of a stable acyl enzyme within 15 min, and excellent in vivo efficacy), the chemical features of these investigational penems gives impetus for future clinical development. As such, compounds with these properties may make a significant impact on the development of "second-generation" inhibitors that target resistant β-lactamases.

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