Novel Insights into the Mode of Inhibition of Class A SHV-1 β-Lactamases Revealed by Boronic Acid Transition State Inhibitors

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Boronic acid transition state inhibitors (BATSIs) are potent class A and C β-lactamase inactivators and are of particular interest due to their reversible nature mimicking the transition state. Here, we present structural and kinetic data describing the inhibition of the SHV-1 β-lactamase, a clinically important enzyme found in Klebsiella pneumoniae, by BATSI compounds possessing the R1 side chains of ceftazidime and cefoperazone and designed variants of the latter, compounds 1 and 2. The ceftazidime and cefoperazone BATSI compounds inhibit the SHV-1 β-lactamase with micromolar affinity that is considerably weaker than their inhibition of other β-lactamases. The solved crystal structures of these two BATSI s in complex with SHV-1 reveal a possible reason for SHV-1’s relative resistance to inhibition, as the BATSI s adopt a deacetylation transition state conformation compared to the usual acylation transition state conformation when complexed to other β-lactamases. Active-site comparison suggests that these conformational differences might be attributed to a subtle shift of residue A237 in SHV-1. The ceftazidime BATSI structure revealed that the carboxyl-dimethyl moiety is positioned in SHV-1’s carboxyl binding pocket. In contrast, the cefoperazone BATSI has its R1 group pointing away from the active site such that its phenol moiety moves residue Y105 from the active site via end-on stacking interactions. To work toward improving the affinity of the cefoperazone BATSI, we synthesized two variants in which either one or two extra carbons were added to the phenol linker. Both variants yielded improved affinity against SHV-1, possibly as a consequence of releasing the strain of its interaction with the unusual Y105 conformation.

Production of β-lactamases (EC 3.5.2.6) is one of the major mechanisms by which bacteria develop resistance to β-lactam antibiotics. In nature, four classes of β-lactamase enzymes exist (classes A to D). Classes A, C, and D are serine-based β-lactamases, while class B uses a metal ion (Zn2+) to hydrolyze the lactam bond. TEM-1 and SHV-1 β-lactamases are among the most commonly observed class A β-lactamases found in Escherichia coli and Klebsiella pneumoniae. As such, this family of β-lactamases presents a significant clinical threat (3, 21).

To counteract β-lactamases, mechanism-based inhibitors were developed to be administered in concert with β-lactam antibiotics (9). Presently, there are three commercially available β-lactamase inhibitors (clavulanate, sulbactam, and tazobactam) which are effective primarily against class A β-lactamases (Fig. 1). Unfortunately, bacteria possessing β-lactamase enzymes have adapted under continued drug pressure and some variants of class A β-lactamases are now “inhibitor resistant” (9). Moreover, class C and D enzymes are poorly inhibited by any of the three commercial inhibitors. Therefore, there is an urgent need to develop novel β-lactam antibiotics and new types of β-lactamase inhibitors to counteract the current crisis in antimicrobial resistance (9).

Based on properties characteristic of the boron atom, boronic acid transition state inhibitors (BATSIs) were developed as serine β-lactamase inhibitors (6, 11). By being competitive and reversible inhibitors, boronates also offer an opportunity to study the reaction coordinate (mechanism) of a β-lactam (26) interacting with a β-lactamase.

In the development of these active-site probes, one approach uses the R1 side chains of potent β-lactam antibiotics and combines them with a boronic acid moiety with the goal of mimicking the transition state and creating a high-affinity, reversible inhibitor that cannot be inactivated by β-lactamases due to not bearing the hydrolyzable β-lactam ring (24). In this context, the efficacy and mechanism of inhibition of BATSI s have been explored against a variety of clinically important β-lactamases. Most of the studies have been focused on TEM-1 and AmpC β-lactamases, with a few studies exploring the inhibition of the CTX-M family as well (5, 16, 22). Although a number of functional studies have been performed on the mechanism of inhibition of BATSI s against inhibitor-resistant and extended-spectrum SHV β-lactamases (8, 25, 26), structural analyses have not yet fully complemented these previous investigations, which is the primary focus of this study.

We present kinetic and crystallographic data describing different BATSI s inhibiting SHV-1 β-lactamases. These BATSI s include a ceftazidime BATSI and a cefoperazone BATSI, each containing the R1 side chain of its respective β-lactam antibiotic. In addition, we have synthesized and characterized structure-based derivatives of the latter BATSI. These new BATSI s SHV-1 structures and BATSI derivatives expand our understanding of the mechanism of inhibition against a clinically
important family of β-lactamases and yield gains and insights into future β-lactamase inhibitor design.

MATERIALS AND METHODS

Synthesis of boronic acid inhibitors. 1H and 13C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX-200 or Avance 400 spectrometer. The chemical shifts (δ) are reported in parts per million downfield from the internal standard, tetramethyl silane (TMS) (s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet). Coupling constants (J) are recorded in hertz. Optical rotations were recorded at 20°C on a PerkinElmer 241 spectropolarimeter, and specific rotations are given in 1015 degrees cm2 g−1. For elemental analyses, a Carlo Erba elemental analyzer (model 1110) was used. All the BATSIs were synthesized as pinacol esters that spontaneously hydrolyze in the phosphate buffer, generating the corresponding boronic acids. The ceftazidime BATSI and cefoperazone BATSI analogues (Fig. 1) were synthesized as previously described (7). Compounds 1 and 2 were obtained in two steps according to the scheme shown in Fig. 1B by using the appropriate amino acids (D-homotyrosine, synthesized as hydrobromide from D-aspartic acid [15, 28], and commercially available D-tyrosine, respectively), commercially available 4-ethyl-2,3-dioxopiperazine-1-carbonyl chloride, and bis-(trimethylsilyl)-aminomethaneboronate, synthesized as previously described (7).

Synthesis of acids A and B. A solution of 2 M NaOH (2 mmol for amino acid A and 1 mmol for amino acid B) and 1.1 mmol of NaHCO3 were sequentially added to a stirred solution of each amino acid (1 mmol) in freshly distilled dioxane (5 ml). The reaction mixture was cooled to 0°C, and commercially available dioxo-piperazine-1-carbonyl chloride (1.1 mmol) was added as a solid. The mixture was stirred for 15 min at 0°C and 2 h at room temperature and then was partitioned between water (10 ml) and ethyl acetate (10 ml). The aqueous phase was acidified to pH 2 to 3 using 10% HCl and extracted 3 times with ethyl acetate (10 ml). The organic layers were dried (MgSO4), filtered, and concentrated in vacuo to afford the desired product, which was promptly used for the next step without further purification.

For (R)-2-[(4-ethyl-2,3-dioxo-piperazine-1-carbonyl)-amino]-4-(4-hydroxyphenyl)-butyric acid A, yield 38%. [α]D20 = −13.1 (c 1.4%, tetrahydrofuran). 1H NMR (200 MHz, acetone-d6): 1.18 (3H, t, J = 7.1 Hz, CH2C6H5), 2.01 to 2.23 (2H, m, CH2), 2.63 (2H, t, J = 7.7 Hz, CH2), 3.52 (2H, q, J = 7.2 Hz, C6H2CH3), 3.66 to 3.78 (2H, m, CH2 piperazine), 4.02 to 4.13 (2H, m, CH2 piperazine), 4.49 (1H, dt, J = 7.5, 5.1 Hz, CH), 6.77 (2H, d, J = 8.6 Hz, meta), 7.03 (2H, d, J = 8.6 Hz, ortho), 9.40 (1H, d, J = 7.5 Hz, NH). 13C NMR (50 MHz, acetone-d6): δ 11.5, 30.5, 34.0, 40.6, 42.0, 43.3, 53.1, 113.8, 115.2, 129.3, 131.6, 152.8, 155.7, 156.4, 159.6, 172.3.

For (R)-2-[(4-ethyl-2,3-dioxo-piperazine-1-carbonyl)-amino]-3-(4-hydroxyphenyl)-propionic acid B, yield 29%. [α]D20 = 1.5 (c 1.1%, CH3OH). 1H NMR (400 MHz, acetone-d6): δ 1.18 (3H, t, J = 7.1 Hz, CH2OH), 3.05 (2H, d, J = 7.1, 14.0 Hz, CH2), 3.16 (1H, dd, J = 7.1, 14.0 Hz, CH2), 3.51 (2H, q, J = 7.2 Hz, CH2CH3), 3.63 to 3.75 (2H, m, CH2 piperazine), 4.66 (1H, dt, J = 5.1, 7.2 Hz, CH3), 6.78 (2H, d, J = 8.6 Hz, meta), 7.10 (2H, d, J = 8.6 Hz, ortho), 9.31 (1H, d, J = 7.2 Hz, NH). 13C NMR (100 MHz, acetone-d6): δ 8.115, 36.6, 40.5, 42.0, 43.3, 55.2, 115.3, 127.1, 130.4, 152.8, 155.7, 156.4, 159.5, 171.7.
Synthesis of compounds 1 and 2. N-Methylphosphonic acid (1 mol) and isobutyl chloroformate (1 mol) were added to a solution of acid A or B (1 mol) in anhydrous tetrahydrofuran (THF) (10 ml) at −35°C and allowed to react under argon atmosphere for 1 h. A solution of bis-(trimethylsilyl)-aminomethaneboronate (1.05 mmol) in anhydrous THF (4 ml) previously treated for 30 min with anhydrous methanol (1.05 mmol), was added at the same temperature. After 1 h, the cooling bath was removed and the mixture was allowed to react 2 to 3 h at room temperature. Thereafter, the precipitate was removed by centrifugation, the supernatant was concentrated, and the compound precipitated as a solid with dry diethyl ether.

For (R)-[2-[(4-ethyl-2,3-dioxo-piperazine-1-carboxyl)-amino]-4-(4-hydroxyphenyl)-butanolaminol]-methanoborate 1, yield 55%. mp = 80°C, [α]290 ± 0° (c 0.8, CHCl3). ¹H NMR (400 MHz, CDCl3); 1.22 (3H, t, 7.5 Hz, CH₃), 1.70 to 1.82 (1H, m, CH₂ piperazine), 2.40 to 2.50 (1H, m, CH₂CH₂), 2.52 to 2.61 (3H, m, N-(CH₂)₂), 2.87 to 2.99 (1H, m, CH₃ CH₂), 3.03 to 3.50 (4H, m, CH₂CH₂ piperazine), 3.93 to 3.98 (2H, m, CH₂ piperazine), 4.50 (1H, m, CH₃), 6.73 (2H, d, J = 8.4 Hz, meta), 6.94 (2H, d, J = 8.4 Hz, ortho), 8.08 (1H, br, NH), 8.73 (1H, d, J = 7.5 Hz, NH). OH not seen. ¹³C NMR (100 MHz, CDCl3); 22.1, 29.8, 30.0, 36.2, 36.8, 37.4, 40.7, 42.3, 43.6, 54.0, 82.3, 115.9, 125.7, 130.6, 152.6, 155.7, 156.0, 158.0, 175.1. C₂₃H₂₂BrN⁴O₇: calculated. C 57.38, H 7.26, N 10.91.

For (R)-[2-[(4-ethyl-2,3-dioxo-piperazine-1-carboxyl)-amino]-3-(4-hydroxyphenyl)-propionylamino]-methanoborate 2, yield 30%. mp = 151°C, [α]290 ± 59.0° (c 0.8, CHCl₃). ¹H NMR (400 MHz, CDCl3); 1.17 (3H, t, 7.1 Hz, CH₃CH₂), 1.31 (2H, s, pinacol CH₂), 2.52 (1H, d, J = 15.1 Hz, NCH₂B), 2.59 (1H, d, J = 15.1 Hz, NCH₂B), 3.03 (1H, dd, J = 14.0, 5.7 Hz, CHB), 3.17 (1H, dd, J = 14.0, 5.7 Hz, CHB), 3.38 to 3.61 (4H, m, CH₂CH₂CH₂, piperazine), 3.82 to 4.12 (2H, m, CH₂ piperazine), 4.78 (1H, m, CH₃), 6.76 (2H, d, J = 7.9 Hz, meta), 7.01 (2H, d, J = 7.9 Hz, ortho), 7.94 (1H, br, NH), 8.85 (1H, br, OH), 8.95 (1H, d, J = 7.5 Hz, NH). ¹³C NMR (100 MHz, CDCl3); 21.1, 24.8, 25.0, 30.0, 36.5, 40.6, 42.7, 43.6, 54.0, 82.3, 115.9, 125.7, 130.6, 152.6, 156.0, 156.5, 159.0, 174.4, C₂₂H₂₁BrNO₇: calculated. C 56.57, H 6.81, N 11.47; found C 56.80, H 7.07, N 11.30.

Enzyme purification. The SHV-1 β-lactamase was expressed and purified as a fusion protein with 20 μg/ml chloramphenicol. Cells were pelleted and lysed by stringent conditions. Anhydrous methanol (1.05 mmol), was added at the same temperature. Thereafter, the precipitate was removed by centrifugation, the supernatant was concentrated, and the compound precipitated as a solid with dry diethyl ether.

Kinetics. Steady-state kinetics were determined on an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). Each continuous assay was performed in 10 mM phosphate-buffered saline (PBS) (pH 7.4) at room temperature. The model for the reaction of an SHV-1 β-lactamase with a BATSI can be presented as follows:

\[
k_i \quad \text{E} + \text{I} \rightleftharpoons k_2 \quad \text{EI} \rightleftharpoons k_3 \quad \text{EI}^{*+} \rightleftharpoons k_i \quad \text{E} + \text{I}
\]  

Here, \( k_i \) represents the β-lactamase, \( I \) is the BATSI, \( EI \) is the Michaelis-Menten complex, \( EI^{*+} \) is the acylation transition state, and \( E^{*+} \) is the deacylation transition state. \( K_i \) values were calculated by measurement of the initial velocity \( (v) \) in the presence of a constant concentration of enzyme, and increasing concentrations of the boronate inhibitors \( I \) competed against the inhibitor substrate nitrocefin (NCF; BD Biosciences, San Jose, CA) (ΔA₆₅₀ = 17,400 M⁻¹ cm⁻¹). The \( K_i \) values were corrected to account for the affinity of NCF for SHV-1 as described by Drewz et al. (7) and are shown in Table 1.

Crystallographic and soaking. Crystals were grown at 20°C using the vapor diffusion sitting-drop technique (13). A 5-μl drop was prepared using 2.0 μl protein solution, 0.5 μl 5.6 mM Glycine-6 (Hampton Research), and 2.5 μl reservoir solution (20 to 30% polyethylene glycol 6000 [PEG 6000] in 100 mM HEPES, pH 7.0) and equilibrated over a 1-ml reservoir solution. Crystals of SHV-1 grew in 2 to 3 days. SHV-1 β-lactamase crystals were soaked with mother liquor containing a 10% (vol/vol) saturated ceftazidime BATSI for 1 h to obtain the ceftazidime BATSI-bound structure. To obtain the cephaloridine BATSI-bound structure, crystals were soaked in mother liquor containing 30 mM cephaloridine BATSI overnight. The same was done for the cephaloridine BATSI analogues except that the soaking concentration of compound 1 was 20 mM and that of compound 2 was 25 mM. The soaked crystals were cryoprotected with 20 to 25% 2-methyl-2,4-pentanediol (MPD) in mother liquor containing the corresponding inhibitor and flash frozen in liquid nitrogen prior to data collection.

Data collection and structure determination. X-ray diffraction data for the SHV-1-ceftazidime BATSI complex was collected at the Advanced Photon Source 19-ID. Data for the cephaloridine BATSI complex was collected at the National Synchrotron Light Source X-29. Data for the compound 1 complex and compound 2 complex were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) beam line 7-1. All of the data were processed using HKL2000 (19). Structures were determined using the isomorphous crystal structure of wild-type (wt) SHV-1 β-lactamase complexed with tazobactam (Protein Data Bank [PDB] identification [ID] number 1VM1) (tazobactam and waters were removed before refinement) (12). Refinement was carried out using REFMAC (17), and model building was done using Coot (10). After initial refinement, strong density in the active site was observed for each of the respective BATSIs. The PRODRG2 server (23) was used to obtain the parameter and topology files for the four BATSIs. In the SHV-1-ceftazidime BATSI complex structure, additional electron density was present in between two neighboring crystallographically related protein molecules representing an intact cephaloridine BATSI molecule esterified with MPD. MPD was present in trace amounts in the pinacol used as a protecting group for the synthesis of the cephaloridine BATSI and ultimately leading to the formation of this six-membered cyclic ester (a slowly hydrolyzable boron ring structure that was less than 5% detectable by ¹H NMR in the cephaloridine BATSI). Crystallographic refinement was monitored using the program DDOQ (27), and the final model quality was assessed using PROCHECK (14). Data collection and refinement statistics are shown in Table 2.

Protein structure accession numbers. Coordinates and structure factors for the SHV-1 complexes with cephaloridine BATSI, cephaloridine BATSI, compound 1, and compound 2 have been deposited with the PDB under accession numbers 3MKE, 3MKF, 3MXR, and 3MXS, respectively.

RESULTS

Our kinetic studies on SHV-1 showed a significant difference between the \( K_i \) values of ceftazidime BATSI and those previously reported for other β-lactamases (Table 1). With respect to ceftazidime BATSI, the lowest \( K_i \) was observed against AmpC (20 nM) and CTX-M-9 (15 nM), whereas the \( K_i \) against SHV-1 was about 100-fold higher (\( K_i \) = 2.2 μM). We interpret these differences to mean that despite significant protein sequence homologies among the β-lactamases and possible differences in the evaluation of kinetic constants, the detailed interactions of the active sites of these enzymes are likely significantly different and improvement of these BATSIs are needed to attempt to arrive at BATSIs with inhibitory potency against a broader array of β-lactamases.

The crystal structures of cephaloridine BATSI, cephaloridine BATSI, compound 1, and compound 2 in complex with class A SHV-1 β-lactamases were determined to have resolutions of 1.75 Å, 1.33 Å, 1.30 Å, and 1.24 Å, respectively (Table 2).
initial unbiased omit $|F_o| - |F_c|$ map of each structure revealed a covalently attached ligand to the Oγ atom (side chain oxygen) of Ser70 of the SHV-1 β-lactamase with a single con-
former (Fig. 2A, B, D, and E). Including the respective gen-
(1) of Ser70 of the SHV-1 β-lactamase structure (Fig. 3A), a tetrahedral geometry is observed around the boron atom, as expected for a transition state analog inhibitor. The O-1 atom of the ceftazidime BATSI occupies the oxyanion hole as it is hydrogen bonded to the backbone nitrogen atoms of S70 and A237; the backbone oxygen atom of A237 is also interacting with the O-1 atom. The O-2 atom of the ceftazidime BATSI is observed interacting with the side chains of E166 and N170, and this oxygen atom is thus residing in the space usually occupied by the deacylation water molecule present in class A β-lactamases. These observations indicate that the ceftazidime BATSI is in a deacylation transition state conformation (18) when bound to the SHV-1 β-lactamase. The amide moiety of the ceftazidime BATSI is interacting with SHV-1 via the interaction between the O-6 atom of the ceftazidime BATSI and the side chain nitrogen atom of N132. The carboxylate of the dimethyl carboxyl moiety of the ceftazidime BATSI resides in the carboxylate binding cavity conserved for the Cδ carboxylate of the cephalosporin (defined by S130, K234, T235, and R244 [20] of class A β-lact-
amases) forming a salt bridge with R244 and hydrogen bonds with residues T235, S130, and K234. In addition, the ceftazidime BATSI also makes a ring-stacking interaction with Y105 via the ceftazidime BATSI’s thiazole ring. This allows the dimethyl carboxyl linker moiety to fold back into the active site and reside in the carboxyl binding pocket as mentioned above. Finally, the ceftazidime BATSI forms an intramolecular hydrogen bond of the amine nitrogen N4 with atom O-15.

Comparing the active sites of the ceftazidime BATSI complex structure with that of apo SHV-1, the following protein atoms have shifted relative to the center of the active site (Fig. 4A). In the SHV-1 structure, residue E166 moved in toward the ceftazidime BATSI (0.6 Å) and N170 moved away from it.

### TABLE 2. Data collection and refinement statistics

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<th>Parameter</th>
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<th>Cefoperazeon BATSI</th>
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* Values in parentheses refer to the highest-resolution shell.
so that both are now within strong hydrogen-bonding distance of O-2 of the ceftazidime BATSI. Moreover, S130 moved in 0.4 Å and T235 moved in 0.5 Å to stabilize the carboxylate tail of the ceftazidime BATSI. We also observed that Y105 moved inwards 0.5 Å and shifted laterally slightly to allow stacking with the thiazole ring of the ceftazidime BATSI. Residue A237 moved inwards 0.3 Å to stabilize the O-1 atom of the ceftazidime BATSI. Shifts less than 0.2 Å are not discussed since they are close to the coordinate error.

**Cefoperazone BATSI structure.** The cefoperazone BATSI adopts a deacylation transition state conformation in SHV-1 β-lactamase as it adopts the same tetrahedral geometry around the boron atom with positions and interactions of the O-1 and O-2 atoms similar to those of the ceftazidime BATSI (Fig. 3B). The amide moiety of the cefoperazone BATSI is observed interacting with the main chain carbonyl oxygen of A237 and the side chain nitrogen atom of N132. The cefoperazone R1 side chain is partially pointing toward the bulk solvent, yet the phenol moiety of the cefoperazone BATSI still makes favorable end-on stacking interactions with Y105, whereas the O-20 and N-23 atoms of cefoperazone BATSI hydrogen bond to the side chain hydroxyl group of T167. Finally, there is a water-mediated interaction between O-22 of the cefoperazone BATSI and the hydroxyl group of Y105.

Comparing the active sites of the cefoperazone BATSI complex structure with that of apo SHV-1, we see the following protein atoms moving upon binding the cefoperazone BATSI (Fig. 4A). Like with the ceftazidime BATSI complex, residue E166 is shifted inwards 0.6 Å toward the cefoperazone BATSI so that it is now in strong hydrogen-bonding distance with the O-2 atom of the cefoperazone BATSI. Co of A237 moved in 0.3 Å such that carbonyl oxygen of A237 is now in hydrogen-bonding distance with the N,H of the cefoperazone BATSI, whereas Y105 flipped 115° to make end-on stacking with the piperazine ring of the cefoperazone BATSI. Finally, residues S130 and T235 moved in 0.5 Å, although they provide no direct interactions with the cefoperazone BATSI.

**Compound 1 and compound 2 structures.** Compound 1 and compound 2 are analogs of the cefoperazone BATSI, and both were designed to maintain favorable interactions (i.e., with the piperazine-containing ring) yet lessen the impact that the parent BATSI has on the Y105 conformation, which is in the swung-out conformation in the starting structure of the cefoperazone BATSI. An additional design consideration was to
FIG. 3. Stereo view of interactions of the bound ligands within the SHV-1 β-lactamase active site. (A) Ceftazidime BATSI; (B) cefoperazone BATSI; (C) compound 1; (D) compound 2. Dashed black lines indicated hydrogen bonds. wat, water.
increase the length of the phenol moiety such that future modifications of this aromatic ring can include a carboxyl moiety that can reach the conserved carboxyl binding pocket in β-lactamases. Our structure-based design yielded compounds 1 and 2, which have two and one additional methyl group, respectively, in the phenol linker, with the aim to make the phenol moiety more flexible such that the end-on stacking interactions with the swung-out Y105 conformation would be disfavored. The structures of BATSI compounds 1 and 2 reveal that both compounds adopt a deacylation transition state conformation in the SHV-1 β-lactamase similar to that of the parent BATSI. The major portion of the designed BATSI adopts the same conformation as the parent cepofazone BATSI. There are some minor differences in the position of the piperazine-containing moiety, although this moiety is largely in the same location (Fig. 4G). However, the phenol moieties of compound 1 and compound 2 are less ordered and largely in the same location (Fig. 4G). Both Y105 and the phenol moiety of the two compounds display flexibility, as shown in the omit |Fo| - |Fc| (Fig. 2D and E). Both compounds have an increased affinity compared to the parent BATSI, with compound 2 having an ~2-fold improvement of the Kᵢ value (Table 1).

**DISCUSSION**

The four BATSI SHV-1 complex structures offer key insights into inhibition of the SHV-1 β-lactamase. We compared the ceftazidime BATSI–SHV-1 structure with previously determined structures of the ceftazidime BATSI in complex with TEM-1, AmpC, and CTX-M (Fig. 4B). The active-site superposition reveals that the ceftazidime BATSI adopts similar conformations in the active sites of TEM-1, AmpC, and CTX-M β-lactamases but is in a different conformation when bound in the active site of the SHV-1 β-lactamase. These striking differences in BATSI orientation are especially surprising since SHV-1, CTX-M, and TEM-1 are class A enzymes and share significant sequence identities and have very similar active sites (superpositioning of 11 active-site residues of the ceftazidime BATSI–SHV-1 structure with those in the ceftazidime BATSI–TEM-1 structure results in an RMSD of 0.299 Å for Cα atoms) (Fig. 4B). Since *Escherichia coli* AmpC is a class C β-lactamase, in which more substantial differences could be expected due to the lower sequence identity and larger active-site differences, we will compare our SHV-1 complex to the more similar class A TEM-1 and CTX-M β-lactamases. This analysis points to five major differences.

First, in the complexed TEM-1 and CTX-M-9 structures, the ceftazidime BATSI behaves as an acylation transition state inhibitor, with one of the boronic acid oxygen atoms in the oxynion hole and the other taking the extrapolated position of the lactam nitrogen in the anticipated Michaelis-Menten complex. In contrast, the ceftazidime BATSI–SHV-1 complex reveals that the ceftazidime BATSI is bound in a deacylation transition state conformation, with one of the boronic acid oxygen atoms occupying the deacylation water pocket (Fig. 4B to D). Second, while in the complexed TEM-1 and CTX-M structures, the torsion angle of atoms O-6, C-5, C-7, and N-14 is clinal while in the SHV-1 structure this angle is periplanar. Third, in the complexed TEM-1 and CTX-M structures, the ceftazidime BATSI’s amide nitrogen makes a weak hydrogen bond with the main chain carbonyl oxygen of A237, while in the SHV-1 complex the 4.4-Å distance between these two atoms is too great to facilitate a hydrogen bond. Fourth, the thiazole ring of the ceftazidime BATSI in TEM-1 and CTX-M is oriented toward and interacts with the side chain of E/D240 via its amino group. In the SHV-1 structure, the ceftazidime BATSI’s thiazole ring adopts an orientation different from that seen in the TEM-1 and CTX-M structures, and this moiety is stabilized mainly by the ring-stacking interaction with Y105. Finally, in complex with TEM-1 and CTX-M, the ceftazidime BATSI’s carboxylate tail points toward the bulk solvent and is stabilized mainly by water-mediated hydrogen bonds to the other parts of the inhibitor molecule (Fig. 4). In contrast, in the SHV-1 complex structure, the ceftazidime BATSI’s carboxylate tail folds back into the active site by positioning itself in the β-lactam carboxylate binding pocket (comprised of R234, T235, S130, and K244) in close proximity to the boron head.
group. Taken together, these differences indicate that the ceftazidime BATSI adopts very different orientations in the enzyme active sites of the two structures. These differences of ceftazidime BATSI conformation are accompanied by several subtle active-site differences between the TEM-1/CTX-M-9–ceftazidime BATSI complexes and the SHV-1–ceftazidime BATSI complex structure. The active-site differences between these class A β-lactamases include (i) a movement of E166 (Ca shift of 0.7 Å) toward the ceftazidime BATSI to make a hydrogen bond with one boronic acid hydroxyl group of the ceftazidime BATSI and (ii) a movement of A237 (Ca shift of 0.5 Å) such that it can no longer form a hydrogen bond with N4 of the ceftazidime BATSI (Fig. 4E). In the SHV-1 structure, N170 moves away from the ceftazidime BATSI to make space for the second boronic acid hydroxyl group of the ceftazidime BATSI and to enable a strong hydrogen-bonding interaction (2.6 Å).

In complex with TEM-1 and CTX-M, the second boronic acid hydroxyl group of the ceftazidime BATSI takes the position close to the hydroxyl group of S130 and is stabilized by two water molecules. The position of this second hydroxyl group of the ceftazidime BATSI is likely one of the steric reasons the carboxylate moiety of the ceftazidime BATSI in the TEM-1/CTX-M structures cannot occupy the carboxylate binding pocket the way the ceftazidime BATSI does in the SHV-1 structure.

Interestingly, the ceftazidime BATSI adopts two different orientations in the seemingly very similar TEM-1 and SHV-1 β-lactamases. Considering the entropic relationships evident here, it would seem energetically favorable for the ceftazidime BATSI to occupy the deacylation water pocket, as it would release an ordered water molecule, thereby increasing entropy upon ceftazidime BATSI binding (18). Nevertheless, the $K_i$ values (Table 1) suggest that SHV-1 is an “outlier” in that it is not as readily inhibited by BATSIs as TEM-1 and AmpC. Although the ceftazidime BATSI is a broad-spectrum inhibitor of both class A and class C β-lactamases, it is about 10-fold more potent against TEM-1 and Acinetobacter-derived cephalosporinase (ADC) and about 100-fold more potent against CTX-M-9 and AmpC β-lactamases than against SHV-1 (Table 1). A similar trend is also present for the cefoperazone BATSI, as its inhibition of SHV-1 is also less potent than that of ADC (Table 1). Note that although the ceftazidime BATSI has an unreactive 6-membered boron ring analog of the ceftazidime BATSI present in solution, as evidenced by the electron density (Fig. 2B), this species exists only in trace amounts (less than 5%) and so it should have little effect on the $K_i$ value and no effect on true ceftazidime BATSI binding in the active site. A possible reason for SHV-1 to be less inhibited by BATSIs could be that both the ceftazidime BATSI and cefoperazone BATSI resulted in the deacylation transition state conformation in SHV-1 while other class A β-lactamases have BATSIs in the acylation transition state (Fig. 4F). Note that adopting this deacylation transition state conformation does not necessarily indicate weaker inhibition, as the chiral penicillin BATSI analogues, among the most potent BATSIs, are also observed to be in the deacylation transition state conformation (Fig. 4F) (18, 24). Furthermore, the potent chiral cephalothin BATSI is observed to be in the deacylation transition state conformation (Fig. 4F) (5).

Why does the ceftazidime BATSI adopt a deacylation transition state conformation? In our crystal structure, we observe that the carboxyl oxygen of A237 in the complex form is positioned deeper into the active site in SHV-1 than in the TEM-1 and CTX-M-9 complexes. In fact, this is also true when the apo structures of SHV-1 and TEM-1 are superimposed, which indicates that the retracted position of the A237 carboxyl in SHV-1 is not induced by binding of the ligand. This carboxyl oxygen is in close proximity (<3 Å) to the boronic acid oxygen atom that occupies the oxyanion hole. Therefore, the A237 carboxyl oxygen, depending on its position, could direct the way in which the BATSIs are attacked by the S70 residue or influence the repositioning of the boron oxygen atoms, via rotation around the boron—serine bond, after the BATSI is covalently attached to S70. Alternatively, the different conformations of the BATSIs could also be due to the much higher ionic strength and pH that was needed to obtain the TEM-1/CTX-M-9 structures (4, 5, 16, 22).

The SHV-1–cefoperazone BATSI structure reveals a deacylation transition state conformation of the boronic acid oxygen atoms similar to that observed in the SHV-1–ceftazidime BATSI structure (Fig. 4F). The amide group of the cefoperazone BATSI interacts with both N132 and the carbonyl atom of A237 in the same fashion observed in some of the TEM-1– and CTX-M-9–BATSI complexes. An interesting feature of the cefoperazone BATSI complex is that residue Y105 of SHV-1 rotates out such that the phenol moiety of the cefoperazone BATSI can provide an end-on interaction with Y105. Such end-on stacking between two aromatic groups was found to be energetically favorable (2). This suggests that the conformation of Y105 is flexible to a certain degree, which can be taken into account for future BATSI design (1). In addition to the orientation of Y105, the positions of the boronic acid hydroxyls are another parameter that can potentially be altered by changing the substituents of the BATSI compounds and optimized in the design of future, more potent BATSIs. To optimize the cefoperazone BATSI for improved inhibitory potency against SHV-1 and other β-lactamases, we modified the BATSI such that the favorable active-site interactions with the piperazine-containing moiety were left intact while the BATSI’s phenol linker length was increased. The reasoning for the latter modification was (i) to weaken the phenol moiety’s end-on stacking interaction with Y105 in the displaced conformation and (ii) so that the increased linker length of the phenol moiety could aid future iterations of this BATSI such that a carboxyl-containing moiety can be added to this aromatic ring that might reach the conserved carboxyl binding pocket. Utilization of this β-lactamase carboxyl binding pocket by BATSIs has led to some of the most potent BATSIs (Fig. 4F) (18, 24). Our design goal was in part successful for several reasons. First, both new BATSI compounds 1 and 2 held the Y105 side chain less rigidly in place in the outward conformation as two weaker occupied Y105 positions were observed (Fig. 2D and E). Second, both designed BATSIs have improved inhibitory potencies, likely a result of the release of this strained interaction that caused a partial distortion of the active site via repositioning of Y105 in the cefoperazone BATSI complex. Third, for future BATSI design, molecular modeling using the compound 1 BATSI structure indicates that the extended and more flexible phenol moiety can be rotated toward
the active site such that an additional carboxyl moiety added to the aromatic ring could reach the conserved carboxyl pocket. This and other modifications will be targeted in our future studies.

In summary, we have determined the crystal structures of four BATSI compounds bound to SHV-1. Our results indicate that the conformations of the R1 side chains as observed in our four BATSI compounds bound to SHV-1. Our results indicate studies. This and other modifications will be targeted in our future studies. The aromatic ring could reach the conserved carboxyl pocket. The active site such that an additional carboxyl moiety added to the corresponding substrates, something that has been previously noted (4). The importance of these differences remains to be explored.

Our structures also point to interesting differences between the BATSI conformations when bound to SHV-1 and those observed with identical and similar BATSI compounds bound to related class A β-lactamases. As drug-resistant enzymes continue to evolve, the number of parameters involved in the design of novel inhibitors will continue to grow. We have characterized several different specific interactions between ceftazidime and cefoperazone BATSI and SHV-1 that can be used as additional pieces of scaffolding in the framework for the further development and optimization of new BATSI compounds targeting β-lactamases (9) and that will hopefully lead to more potent and increasingly effective broad-spectrum β-lactamase inhibitors.

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