Boronic Acid Transition State Inhibitors Active against KPC and Other Class A β -Lactamases: Structure-Activity Relationships as a Guide to Inhibitor Design

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Boronic acid transition state inhibitors (BATSI)s are competitive, reversible β -lactamase inhibitors (BLIs). In this study, a series of BATSI)s with selectively modified regions (R1, R2, and amide group) were strategically designed and tested against representative class A β -lactamases of *Klebsiella pneumoniae*, KPC-2 and SHV-1. Firstly, the R1 group of compounds 1a to 1c and 2a to 2e mimicked the side chain of cephalothin, whereas for compounds 3a to 3c, 4a, and 4b, the thiophene ring was replaced by a phenyl, typical of benzylpenicillin. Secondly, variations in the R2 groups which included substituted aryl side chains (compounds 1a, 1b, 1c, 3a, 3b, and 3c) and triazole groups (compounds 2a to 2e) were chosen to mimic the thiazolidine and dihydrothiazine ring of penicillins and cephalosporins, respectively. Thirdly, the amide backbone of the BATSI, which corresponds to the amide at C-6 or C-7 of β -lactams, was also changed to the following bioisosteric groups: urea (compound 3b), thiourea (compound 3c), and sulfonamide (compounds 4a and 4b). Among the compounds that inhibited KPC-2 and SHV-1 β -lactamases, nine possessed 50% inhibitory concentrations (IC₅₀s) of ≤ 600 nM. The most active compounds contained the thiopheneacetyl group at R1 and for the chiral BATSI)s, a carboxy- or hydroxy-substituted aryl group at R2. The most active sulfonamido derivative, compound 4b, lacked an R2 group. Compound 2b (S02030) was the most active, with acylation rates (k_2/K) of $1.2 \pm 0.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for KPC-2 and $4.7 \pm 0.6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for SHV-1, and demonstrated antimicrobial activity against *Escherichia coli* DH10B carrying *bla*_{SHV} variants and *bla*_{KPC-2} or *bla*_{KPC-3} and against clinical strains of *Klebsiella pneumoniae* and *E. coli* producing different class A β -lactamase genes. At most, MICs decreased from 16 to 0.5 mg/liter.

The continuous and accelerated emergence of bacterial strains resistant to virtually all antibiotics is one of the most challenging issues in clinical medicine. β -Lactam antibiotics are ancient, and resistance to them can be found at very low levels and is widespread in nature. Unfortunately, the ubiquitous overuse of β -lactams in the past 80 years, at levels massively in excess of those found in nature, has resulted in the rapid, nearly continuous emergence of resistance mechanisms that are new to clinical medicine. Therefore, novel treatment options for infections produced by multidrug-resistant Gram-negative bacteria are required.

Production of β -lactamases is the most common mechanism of resistance to β -lactams in clinically important Gram-negative bacteria (1). Based on conserved amino acid motifs, β -lactamases are grouped into four molecular classes. Class A, C, and D β -lactamases hydrolyze the β -lactam ring and form an acyl enzyme through a serine active site, whereas class B β -lactamases are metalloenzymes characterized by the presence of one or two Zn²⁺ ion binding sites (1). As of 2016, there are roughly 1,800 officially numbered β -lactamases listed on the authoritative website at Lahey Clinic (<http://www.lahey.org/studies>). The astonishing number of β -lactamases reported might be explained by the increasing selective pressure from the proliferation of β -lactam antibiotics as well as advances in the molecular biology tools to detect them (2).

KPCs are versatile class A β -lactamases that threaten the use of all current β -lactams, as KPCs hydrolyze penicillins, cephalosporins, monobactams, and carbapenems and also are resistant to inhibition by clavulanic acid, sulbactam, and tazobactam (3). These carbapenemases, first reported for *Klebsiella pneumoniae* in

2001 (4), are carried by transposons (Tn4401 family) and have disseminated to an increasing number of bacterial genera, becoming the major carbapenemases carried by clinically important Gram-negative organisms worldwide (3, 5).

The SHV family of enzymes belongs to the molecular class A of serine β -lactamases and is found in *K. pneumoniae*, where they originated as narrow-spectrum penicillinases (2). Although the principal localization of *bla*_{SHV} in *K. pneumoniae* is chromosomal, *bla*_{SHV} is also found in many *Enterobacteriaceae* on mobile plasmids (6). Most of the more than 180 plasmid-encoded variants described to date possess extended-spectrum β -lactamase (ESBL) activity, which confers resistance to expanded-spectrum cephalo-

Received 30 October 2015 Returned for modification 15 November 2015

Accepted 27 December 2015

Accepted manuscript posted online 4 January 2016

Citation Rojas LJ, Taracila MA, Papp-Wallace KM, Bethel CR, Caselli E, Romagnoli C, Winkler ML, Spellberg B, Prati F, Bonomo RA. 2016. Boronic acid transition state inhibitors active against KPC and other class A β -lactamases: structure-activity relationships as a guide to inhibitor design. *Antimicrob Agents Chemother* 60:1751–1759. doi:10.1128/AAC.02641-15.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.02641-15>.

For a companion article on this topic, see doi:10.1128/AAC.02643-15.

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FIG 1 BATSIs act by binding to the active site of the enzyme, where they sterically resemble the quaternary transitional state of the β -lactam hydrolysis reaction and occupy the active site with high affinity, leading to inhibition in a reversible competitive manner. Shown are tetrahedral intermediate with cephalothin (top) and BATSIs (bottom).

sporins, such as ceftazidime, as well as monobactams. Some SHV β -lactamases also exhibit resistance to β -lactamase inhibitors (BLIs), such as clavulanate, which further reduces the therapeutic options for treatment of infections caused by bacteria harboring these enzymes (7).

To overcome this growing threat, research is directed toward two strategic goals: (i) to design β -lactam antibiotics that are intrinsically resistant to β -lactamases and (ii) to identify BLIs that would inactivate or inhibit the β -lactamase and allow the partner β -lactam to reach the penicillin binding proteins (PBPs), the primary target of β -lactams (8). Toward the second aim, structure-based design is widely used to discover new BLIs by mimicking interactions observed between the target enzyme and its natural substrates. Of late, great interest has been focused on inhibition of common serine β -lactamases (9). Attention to novel BLIs bearing an electrophilic center (phosphonates, aldehydes, trifluoromethylketones, and boronic acids) that can covalently modify the nucleophilic catalytic serine is conceptually advancing our understanding in this field (10).

Among the novel BLIs bearing electrophilic centers, the boronic acid transition state inhibitors (BATSIs) merit particular attention as clinically important BLIs. BATSIs possess a boron atom acting as an electrophile that mimics the carbonyl carbon of the β -lactam ring and forms a tetrahedral adduct with the catalytic serine that closely resembles a transition state of the hydrolytic mechanism (9, 11, 12). Presently, Carbavance, which is combination of a novel boronic acid-based BLI (RPX7009) with meropenem (RPX2014) for intravenous treatment of hospitalized patients with serious infections, attests to the interest in this class of compounds (e.g., see registration number NCT02168946 at ClinicalTrials.gov).

In a parallel quest, BATSIs were studied and optimized for a variety of β -lactamase enzymes (7, 13–18). Mechanistically, the boronic inhibitors act by binding to the active site of the enzyme, where they sterically resemble the quaternary transition state of the β -lactam hydrolysis reaction and occupy the active site with high affinity, leading to inhibition in a reversible competitive manner (9). Recently, rational inhibitor design efforts were di-

rected toward the inclusion of an R1 side chain on BATSIs that resembles the side groups of known β -lactams (19). This feature is necessary for specific interactions with the β -lactamases. Additionally, BATSIs with a variety of R2 groups were also rationally designed (Fig. 1).

In this study, a series of BATSIs targeting KPC-2, SHV-1, and other class A ESBLs were synthesized and screened. In *K. pneumoniae*, SHV is found on the chromosome and KPC, when found, is plasmid mediated. Thus, an agent that may target specifically *K. pneumoniae* and demonstrate activity against SHV and KPC is of particular interest. A diverse portfolio of agents was designed to explore optimal structure-activity relationships (SAR). In this group of BATSIs, three regions were selectively modified: the R1 group, the R2 group, and the amide group. Our biochemical analysis and antibiotic susceptibility results provide evidence that a strategy can be developed to create effective inhibitions of SHV and KPC β -lactamases based on SAR. Our goal in this study and other studies planned is iterative testing of novel scaffolds to explore the importance of different interactions, not currently known, by the boronic acids in development and to explain their potency by structural studies.

MATERIALS AND METHODS

Synthesis. The BATSIs were synthesized in the desired *R* absolute configuration by stereoselective homologation of (+)-pinandiol boronates. For the triazole-containing BATSIs, a Cu-catalyzed azide-alkyne cycloaddition was performed on the suitable β -azido-boronate (19, 20). A complete description of the organic synthesis of compound 3c is provided in the supplemental material.

Bacterial strains and plasmids. The *bla*_{KPC-2} gene in the pBR322-*catI* vector (pBR322-*catI*-*bla*_{KPC-2}) was a kind gift from the Centers for Disease Control and Prevention (Atlanta, GA) (3). The cloning of *bla*_{SHV-1} into phagemid vector pBC SK(–) was previously described (21). Both plasmids were maintained in *Escherichia coli* DH10B cells.

β -Lactamase purification. The KPC-2 and SHV-1 β -lactamases were expressed and purified as previously described. Briefly, *E. coli* strains harboring SHV-1 and KPC-2 were grown overnight in lysogeny broth (LB) containing 20 μ g/ml of chloramphenicol. Cells were pelleted and lysed by stringent periplasmic fractionation. The soluble fractions were run on a

preparative isoelectric focusing platform as previously described (3, 21). Fast protein liquid chromatography (FPLC) on an AKTA purifier was conducted using a gel filtration Superdex 75 column and anion exchange HiTrapQ XL column. Purity of the fractions was assessed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE). The protein concentrations were determined by measuring the absorbance at 280 nm and Beer's law using the proteins' extinction coefficient ($\Delta\epsilon = 39,545 \text{ M}^{-1} \text{ cm}^{-1}$ for KPC-2 and $\Delta\epsilon = 31,970 \text{ M}^{-1} \text{ cm}^{-1}$ for SHV-1).

Steady-state kinetics. Steady-state kinetics were determined on an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). Each assay was performed in 10 mM phosphate-buffered saline (PBS; pH 7.4) at room temperature.

The inhibition of β -lactamases by BATsIs is hypothesized to follow a slow reversible process represented according to the following scheme:

Here, E represents the β -lactamase enzyme, I represents the BATSI, E:I is the Michaelis complex, E- I^* is the enzyme-inhibitor complex resembling the acylation transition state analog, and E- I^{**} is the deacylation transition state intermediate. This model takes into account the crystallographic intermediates captured in two previous studies (15, 17).

The IC_{50} s were determined after a 5-min preincubation of the β -lactamase (7 nM KPC-2 or 2.5 nM SHV-1) and the BATSI used at increasing concentrations as previously described (19). The initial velocities (v_0) of hydrolysis of the indicator substrate, nitrocefin ($\Delta\epsilon_{482} = 17,400 \text{ M}^{-1} \text{ cm}^{-1}$), were measured, and the data were fit to equation 1.

$$v_0 = \frac{V_{\max} [S]}{K_m \left[1 + \frac{I}{IC_{50}} \right] + [S]} \quad (1)$$

Measurements of apparent association rate constants (k_2/K) for inhibitor binding were carried out in the presence of several concentrations of compound 2b. Reactions were then initiated by the addition of enzyme (2.5 nM KPC-2 or SHV-1) to a mixture of 50 μM nitrocefin and the BATSI, without preincubation. Inhibitor association rate constants were estimated from linear extrapolation of the observed rate constant for inhibition (k_{obs}), which was obtained by fitting progress curves of nitrocefin hydrolysis to equation 2.

$$A = A_0 + v_s t + \frac{(v_0 - v_s)(1 - e^{-k_{\text{obs}} t})}{k_{\text{obs}}} \quad (2)$$

Here A is absorbance, v_0 is the initial velocity, v_s is the steady-state velocity, and k_{obs} is the apparent rate constant for formation of the enzyme-inhibitor complex. The linear dependence of k_{obs} versus concentration of inhibitor [I] at limiting inhibitor concentration approximates second-order rate constant k_2/K . The observed second-order rate constant also includes an adjustment for competition between inhibitor and substrate [S] binding (equation 3).

$$k_{\text{obs}} = k_{-2} + \frac{k_2}{K} \left(\frac{[I]}{1 + \frac{[S]}{K_m}} \right) \quad (3)$$

In order to determine the apparent dissociation rate constant (k_{off}), 1 μM SHV-1 or KPC-2 was incubated with 2.5, 5, 10, or 15 μM compound 2b for 5 min at 37°C and diluted 4,000-fold in the assay buffer (PBS) without inhibitor and 50 μM nitrocefin was added. In the off-rate determination, v_0 represents fully inhibited enzyme velocity and was estimated measuring a reaction without enzyme. Uninhibited enzyme velocity (v_s) was measured using a reaction with SHV-1 and KPC-2 and no added BATSI. The data were fit to equation 2, where k_{obs} was replaced by k_{off} . The k_{off} value is reported as ± 2 standard deviations (SD) from three separate determinations.

Antimicrobial susceptibility testing. To assess the permeability and qualitative antimicrobial activity of compound 2b, the MICs of ceftazidime, cefepime, andertapenem, alone and in combination with 2b, were determined for *E. coli* DH10B carrying pBR322-*catI*-*bla*_{KPC-2} or pBC

SK(-) *bla*_{SHV-1} as well as for clinical isolates containing one or more β -lactamases. The agar dilution method in Mueller-Hinton broth following Clinical and Laboratory Standards Institute (CLSI) recommendations was used (22).

Molecular modeling and docking. The crystal structures of KPC-2 (PDB code 2OV5) and SHV-1 (PDB code 1SHV) were used to generate molecular representations of the interactions of these two β -lactamases with representative BATsIs (achiral cephalothin, 1c, and 2b). Discovery Studio 4.1 (DS 4.1) software (Accelrys, CA) was employed to prepare and optimize the crystallographic structures and to dock the inhibitors into the active site of these enzymes.

Briefly, the minimization was performed in several steps, using the Steepest Descent and Conjugate Gradient algorithms to reach the minimum convergence ($0.001 \text{ kcal mol}^{-1} \cdot \text{\AA}$). The protein was solvated employing a model with periodic boundary conditions. The force field parameters of CHARMM were used for minimization, and the particle mesh Ewald method addressed long-range electrostatics. The bonds that involved hydrogen atoms were constrained with the SHAKE algorithm.

The minimized and equilibrated KPC-2 and SHV-1 structures were used for constructing the complexes of the β -lactamases and the BATSI. The ligand structures were built using DS 4.1 Fragment Builder tools. The molecular docking was performed using CDOCKER module of DS 4.1 (23). The protocol uses a CHARMM-based molecular dynamics (MD) scheme to dock ligands into a receptor binding site. Random ligand conformations were generated using high-temperature MD. The conformations were then translated into the binding site. Candidate poses were then created using random rigid-body rotations followed by simulated annealing. A final minimization was then used to refine the ligand poses.

From the multiple possible conformations generated, the candidate ones were chosen. The complex between the ligand and the enzymes was created, solvated, and energy minimized. The acyl-enzyme complex was created by making a bond with Ser70, and the assembly was further minimized using conjugate gradient algorithm with periodic boundary conditions to 0.001 minimum derivatives.

RESULTS AND DISCUSSION

In this study, we evaluated the SAR of the designed BATsIs against SHV-1 and KPC-2 β -lactamases in biochemical and microbiological assays. The results of kinetics experiments were interpreted using molecular models. After identifying a compound with the most desirable properties, we further performed antimicrobial susceptibility testing and determined microscopic rate constants. We discovered that a BATSI containing a thiophene ring as an R1 substituent and possessing a triazole with a carboxyl group as an R2 side chain demonstrated optimal activity against the KPC and SHV β -lactamases, which are widespread in *Klebsiella* spp.

SAR of BATsIs against KPC-2 and SHV-1 β -lactamases and molecular modeling. After modifying three regions of the BATSI (i.e., the R1 group, the R2 group, and changing the amide group to a sulfonamide, urea, or thiourea), we first explored the structural and affinity contributions of a group of boronic acid inhibitors. Starting with the reference compound, with an amide group and devoid of R1 or R2 side chains, the IC_{50} s were determined to be $\geq 150 \mu\text{M}$ for both SHV-1 and KPC-2 β -lactamases (Table 1). The addition of a thiophene ring at the R1 position (achiral cephalothin) improved the binding to both enzymes, with the greatest increase seen with KPC-2 β -lactamase at $\sim 3 \mu\text{M}$, versus 50 μM for SHV-1. The chiral BATsIs that possessed a cephalothin R1 side chain combined with an amide group and various R2 substituents generally demonstrated greater affinity toward both KPC-2 and SHV-1. Of these thiophene containing BATsIs, seven compounds (1b to 2e) demonstrated IC_{50} s in the nanomolar range (38 to 600 nM). By changing the R1 chain from thiophene to phenyl (com-

TABLE 1 BATSI chemical structures and IC₅₀ data

Compound	Structure		IC ₅₀ (μM)	
	R1	R2	SHV-1	KPC-2
Reference			>200	164 ± 5
Achiral cephalothin			50 ± 2	2.9 ± 0.1
1a			5.9 ± 0.3	2.0 ± 0.2
1b			0.40 ± 0.02	0.60 ± 0.05
1c			0.100 ± 0.001	0.080 ± 0.001
2a			0.130 ± 0.002	0.180 ± 0.002
2b			0.130 ± 0.002	0.080 ± 0.002
2c			0.33 ± 0.02	0.060 ± 0.005
2d			0.43 ± 0.07	0.43 ± 0.07
2e			0.14 ± 0.02	0.04 ± 0.01
3a			0.20 ± 0.05	0.07 ± 0.01
3b			0.90 ± 0.02	0.230 ± 0.002
3c			73 ± 4	32 ± 2
4a			118 ± 7	6.2 ± 0.5
4b			0.43 ± 0.07	0.20 ± 0.01

FIG 2 Molecular docking of achiral cephalothin (a) and compound 1c (b) in the active site of KPC-2. The model of achiral cephalothin (missing the R2 side chain) with KPC-2 shows fewer H-bonding interactions (green dashed lines) compared to those of compound 1c. The boron atom preserves the tetrahedral conformation, as observed in the crystal structures (H-bonding distance with S130, S70:N, and T237:N). The addition of *m*-carboxyphenyl group as the R2 side chain in compound 1c creates interactions with R220. The thiophene ring of 1c is ≈ 3 Å from Trp 105, making possible steric interactions with phenyl ring, which are more favorable than achiral cephalothin ($d > 4$ Å).

pound 1c versus 3a), the IC_{50} s for KPC-2 or SHV-1 were only slightly affected (Table 1).

To understand how the addition of a thiophene ring lowers the IC_{50} s 80-fold compared to the reference compound, a molecular representation of KPC-2 with achiral cephalothin docked in the active site was generated (Fig. 2a). This model suggests that the lack of interactions with the catalytic part of the enzyme may be compensated by the steric interaction of thiophene ring with W105. We advance that since SHV-1 possesses a Tyr at position 105 instead of a Trp, this may explain why it displayed a higher IC_{50} for achiral cephalothin than did KPC-2. In addition, the hydroxyls of the boronate were within hydrogen bond distance of the oxyanion hole atoms, S70:N and T237:N as well as S130. The addition of a phenyl group (compound 1a) at the R2 position improved the IC_{50} for SHV-1 nearly 10-fold (from 50 to 5.9 μ M), suggesting that the addition of an R2 chain was also important for interactions with SHV-1.

Another significant change was observed for the thiophene- and amide-containing BATSIs with the addition of a carboxyl group to the phenyl side chain at the R2 position (compound 1b). The IC_{50} s decreased to 0.4 to 0.6 μ M from 2 to 6 μ M with the phenyl group alone (compound 1a). From our modeling, we hypothesize that the carboxyl group is able to make interactions with R220 (KPC-2) and R244 (SHV-1), as observed with the carboxylate group on β -lactams. By adding an extra carbon (compound

1c) to compound 1b, the IC_{50} s were further lowered to 0.1 to 0.08 μ M, most likely due to improved interactions with positions R220 in KPC-2 and R244 in SHV-1 (Fig. 2b).

The triazole-based R2 side chain present on compounds 2a, 2b, 2c, 2d, and 2e improved the IC_{50} s up to 75-fold and 380-fold for KPC-2 and SHV-1, respectively, compared to those for achiral cephalothin. Regardless of the substituent (carboxyl, phenyl, carboxyphenyl, etc.) attached to the triazole, all of the compounds possessed low IC_{50} s from 0.04 to 0.43 μ M for KPC-2 and 0.13 to 0.43 μ M for SHV-1. Compound 2b was one of the best inhibitors against KPC-2 and SHV-1, with IC_{50} s of 84 ± 2 nM and 130 ± 2 nM, respectively.

To assess the contribution of the triazole moiety, compound 2b was docked into the active sites of KPC-2 and SHV-1. The molecular docking generated multiple conformations for compound 2b (Fig. 3a), suggesting that this compound may demonstrate a high conformational flexibility in the active site of KPC-2 and SHV-1. This finding was consistent with the crystal structure of 2b (S02030) with the class C β -lactamase. ADC-7 revealed multiple conformations of the compound in the active site as well (24). Based on the possible conformations of 2b into the active site of ADC-7, a similar binding mode with KPC-2 and SHV-1 enzymes was chosen (Fig. 3b and c). In this molecular model, the triazole side chain produced a strong dipole moment and behaved as an

FIG 3 Molecular docking of compound 2b into the active sites of KPC-2 (b) and SHV-1 (c). Using high-temperature MD, molecular docking (CDOCKER) generated multiple boronic acid conformations; shown here are 3 of those (gray, yellow, and cyan) (a). The candidate poses (b and c) were chosen and complex enzyme-compound 2b was created. The *m*-carboxyl group attached to the triazole R2 side chain demonstrated interactions with R220 in KPC-2 and R244 in SHV-1. The triazole moiety enhanced binding due to a strong dipole moment and behaved as an active linker in both KPC and SHV enzymes. The hydroxyl boron atoms preserve the interactions with S130 and T237 for KPC-2 (b), but not for SHV-1 enzyme (c). The MD suggests that hydroxyl borons form H bonds with K73, S130, and A237.

FIG 4 Schematic 2D diagram showing possible interactions between BATSI 4d and AmpC β -lactamases (PDB code 3O87) (a) and KPC-2 enzyme (b). Based on the conformational flexibility of compound 4d (c) and the high affinity for KPC β -lactamases, the model suggests that the carboxyl group may interact with residue R220.

active linker by forming interactions with position R220 in KPC-2 and R244 in SHV-1 (Fig. 3a and b).

In the study described in the companion article, the crystal structure of KPC-2 and SHV-1 with 2b (S02030) revealed multiple conformations of 2b in the active site of KPC-2 and SHV-1 (25). The boronic acid preserved the tetrahedral interactions, forming the bond with catalytic serine, positioning one of the boronic acid oxygens in the oxyanion hole (T237:N for KPC-2) and the other toward S130. The amide moiety of 2b interacts with the side chain of N132 as suggested by the molecular model. In the crystal structure, the carboxyl-triazole moiety does not make a salt bridge with either R220 (KPC-2) or R244 (SHV-1) as observed during the MD in the molecular modeling.

When the amide group present in compound 3a was replaced by a urea, thus switching the benzyl R1 group of 3a into an aniline, a slight loss of activity was observed ($0.230 \pm 0.002 \mu\text{M}$ KPC-2 and $0.90 \pm 0.02 \mu\text{M}$ SHV-1 for 3b, compared to $0.07 \pm 0.01 \mu\text{M}$ and $0.200 \pm 0.005 \mu\text{M}$ for 3a, respectively). The loss of activity was much more pronounced after replacement of the amide with a thiourea (compound 3c), as displayed by IC_{50} s of $73 \pm 4 \mu\text{M}$ SHV-1 and $32 \pm 2 \mu\text{M}$ KPC-2 (Table 1), highlighting the importance of the carbonyl as an H bond acceptor.

Compared to achiral cephalothin, the BATSI achiral phenyl sulfonamide (compound 4a) performed similarly against KPC-2 and SHV-1 (Table 1). However, an interesting and unexpected decrease in IC_{50} s was observed after the addition of a carboxyl side chain to the phenyl group (compound 4b). The presence of a carboxyphenyl group at the R1 side chain was beneficial for achiral BATSI. Based on the crystal structure of similar compounds with AmpC β -lactamases (PDB code 3O87) (26) we hypothesize that the carboxyphenyl group will flip to interact with position R220 for KPC-2 and residue R244 for SHV-1 and that the sulfonamide group will interact with N132 (Fig. 4).

Susceptibility testing and biochemical analysis of compound 2b. Among the evaluated BATSI, compound 2b possessed one of the most favorable initial biochemical properties against KPC-2 and SHV-1 and thus was chosen for antimicrobial susceptibility testing and detailed kinetic analysis. We tested the ability of compound 2b to restore susceptibility to cephalothin, cefepime, ceftazidime, and/or ertapenem for *E. coli* DH10B producing KPC-2 and SHV variants, including inhibitor-resistant β -lactamases and ESBLs, as well as clinical isolates with different class A β -lactamases. All strains expressing *bla*_{KPC} enzymes were resistant to cefepime and ceftazidime, while 7 of 8 were resistant to ertapenem (Table 2). When partnered with cefepime or ertapenem, compound 2b restored the susceptibility to all resistant strains carrying *bla*_{KPC} enzymes (Table 2). The ceftazidime-compound 2b combination restored susceptibility to all isolates except *K. pneumoniae* VA375, which displayed an intermediate MIC of 8 mg/liter with the combination. Against a panel of 16 strains expressing *bla*_{SHV}, *bla*_{TEM}, and/or *bla*_{CTX-M} variants, cefepime-compound 2b restored susceptibility to all isolates tested (Table 3). Fourteen of 16 strains were resistant to cephalothin, and when cephalothin was combined with compound 2b, this number decreased to 6 strains. With ceftazidime, 13 of 16 strains demonstrated resistance, but when this drug was combined with compound 2b, only 5 of 16 remained resistant. The cefepime-compound 2b combination was the most effective combination tested against all 24 strains expressing various class A β -lactamases.

To obtain a detailed biochemical profile of compound 2b against KPC-2 and SHV-1, acylation rates (k_2/K) and off-rates (k_{off}) were determined. With the KPC-2 β -lactamase, a k_2/K value of $1.2 \pm 0.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ was obtained, whereas for SHV-1, $4.7 \pm 0.6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ was observed (Fig. 5a and b). These values are comparable to the inactivation efficiency (k_{inact}/K_I) values reported for a variety of other

TABLE 2 MICs for *E. coli* DH10B expressing KPC-2 and clinical strains carrying variety of KPCs with cefepime, ceftazidime, and ertapenem alone and in combination with compound 2b at 4 $\mu\text{g/ml}^a$

Species	Strain	β -Lactamase (s)	MIC ($\mu\text{g/ml}$)					
			FEP	FEP -2b	CAZ	CAZ -2b	ERT	ERT -2b
<i>E. coli</i>	DH10B	None	0.06	0.06	0.5	0.5	0.06	0.06
	DH10B pBR322- <i>catI</i>	KPC-2	16	0.25	128	4	8	0.06
<i>K. pneumoniae</i>	Kpn96	KPC-2, TEM-1	16	0.12	64	2	16	0.5
	KpnST258	KPC-3, TEM, SHV	32	0.5	64	2	4	0.06
	Kpn ST17	KPC-2, TEM, SHV, CTX-M group 1	64	0.12	32	2	8	0.06
	KpnVA375	KPC-3, TEM-1, SHV-11/14	16	0.5	64	8	8	0.5
	KpnVA388	KPC-3 TEM-1, SHV-1	8	0.12	64	2	4	0.06
	EcoPR261	KPC, CTX-M group 1, SHV	16	0.25	64	0.2	16	0.25
<i>E. coli</i>	Eco pLTCF1	CTX-M group 9	8	0.12	8	0.5	0.12	0.06

^a MICs in bold are values that are intermediate or indicate resistance according to the CLSI (33). FEP, cefepime; CAZ, ceftazidime; ERT, ertapenem.

BLIs against class A β -lactamases (e.g., SHV-1, CTX-M-9, and KPC-2) with magnitudes of 10^4 to $10^6 \text{ M}^{-1} \text{ s}^{-1}$ (3, 27–30). Most importantly, the k_2/K values for compound 2b are similar to those of the recently Food and Drug Administration-approved BLI avibactam, with values of $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for KPC-2 and $6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for SHV-1 (31, 32).

The k_{off} values for compound 2b were also determined for KPC-2 and SHV-1, at $0.00046 \pm 0.00002 \text{ s}^{-1}$ and $0.0024 \pm 0.0001 \text{ s}^{-1}$, respectively (Fig. 5c and d). The corresponding residence time half-lives ($t_{1/2} = 0.693/k_{\text{off}}$) were $24.8 \pm 1 \text{ min}$ for KPC-2 and $4.9 \pm 0.5 \text{ min}$ for

SHV-1, respectively. For comparison, the $t_{1/2}$ values for avibactam range from 6 to 300 min for susceptible β -lactamases (i.e., TEM-1, CTX-M-15, KPC-2, PDC-1, and P99) (32).

Conclusion. In this study, novel synthetic strategies were used to investigate three parts of the molecule in the same BATSI. We found that the best R1 side chain was the cephalothin analog. In addition, at position R2, a substituted phenyl or triazole resulted in lower IC_{50} s. Finally, the change of amide into sulfonamide or urea was beneficial (26), while the thiourea was not active. We also discovered that against SHV-1 β -lactamase, and more strikingly

TABLE 3 MICs for *E. coli* DH10B expressing SHV-1 and clinical strains carrying variety of SHV's and other class A β -lactamases with cephalothin, cefepime, and ceftazidime alone and in combination with compound 2b at 4 $\mu\text{g/ml}^a$

Species	Strain	β -Lactamase(s)	MIC ($\mu\text{g/ml}$)					
			CEF	CEF -2b	FEP	FEP -2b	CAZ	CAZ -2b
<i>E. coli</i>	DH10B	None	4	2	0.06	0.06	0.5	0.5
	ATCC BAA-202	SHV-1	128	8	4	0.25	32	8
	ATCC 35218	TEM-1	8	2	0.06	0.06	0.25	0.12
	DH10B pBC SK(–)	SHV-1	128	4	2	0.25	16	2
	DH10B pBC SK(–)	SHV-2	512	4	4	0.06	64	4
	DH10B pBC SK(–)	SHV R244S	4	2	0.12	0.06	1	1
<i>K. pneumoniae</i>	Kpn 266	SHV-5	512	2	4	≤ 0.06	>128	1
	Kpn 104	TEM-1, SHV-1	256	64	4	1	>128	>8
	Kpn 255	TEM-1, SHV-2	256	64	8	2	>128	>8
	Kpn 158	TEM-1, SHV-5	256	32	8	0.5	>128	>8
	Kpn 9	TEM-1, CTX-M-2	1,024	64	16	0.5	32	4
	Kpn 427	TEM-1, SHV-1, CTX-M-3	>1,024	32	8	0.25	16	2
	Kpn 160	TEM-1, SHV-2, CTX-M-3	1,024	16	8	0.5	32	2
	Kpn 59	TEM-1, SHV-5, CTX-M-2	256	4	4	0.12	128	1
	Kpn 238	TEM-12, SHV-2, CTX-M-2	512	32	16	0.5	>128	>8
	Kpn 34700	CTX-M-15, SHV, TEM	>1,024	128	16	1	64	4
<i>E. coli</i>	Eco 29838	CTX-M-14, TEM-1	1,024	32	8	0.25	2	1

^a MICs in bold represent values that are intermediate or indicate resistance according to the CLSI (33). CEF, cephalothin; FEP, cefepime; CAZ, ceftazidime (CAZ).

FIG 5 Inhibition of nitrocefin hydrolysis by compound 2b of KPC-2 (a) and SHV-1 (b). Inhibitor association rate constant k_2/K was estimated from linear extrapolation of the observed rate constant for inhibition, k_{obs} , as function of inhibitor concentration. The dissociation rate, k_{off} , for compound 2b for KPC-2 (c) and SHV-1 (d) represents the recovery of activity as a function of time.

against KPC-2, compound 2b exhibited a time-dependent inactivation, a behavior that was previously observed for other β -lactamases when inactivated with BATSI (19, 24). By identifying common features of these new BATSI, novel SAR studies to target KPC-2 and other clinically important β -lactamases can be performed with the aim of determining key amino acid residues in the catalytic pocket that contribute to molecular recognition. Studies against other important β -lactamases and *in vivo* (animal model) testing are warranted. It has not escaped our attention that benefits and limitations of each new class of BLI must be fully vetted in order to find an optimal inhibitor. Nevertheless, as compounds become more focused and pathogen directed, there is intent to design specific chemotypes to treat particular resistant bacteria (e.g., *K. pneumoniae*). The approach taken in this study is an important step toward “precision medicine.”

ACKNOWLEDGMENTS

We acknowledge the assistance of Focco van den Akker, Department of Biochemistry, Case Western Reserve University School of Medicine, in the preparation of the manuscript.

Research reported in this publication was supported by the Harrington Foundation. This study was supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs to K.M.P.-W. and R.A.B. and the Geriatric Research Education and Clinical Center VISN 10 to R.A.B. The effort of B.S. is also supported by the Harrington Foundation.

The content of this article is solely the responsibility of the authors and

does not necessarily represent the official views of the National Institutes of Health.

FUNDING INFORMATION

HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Robert A. Bonomo under grant numbers R01AI100560 and R01AI063517. U.S. Department of Veterans Affairs (VA) provided funding to Krisztina M. Papp-Wallace under grant number 1I01BX002872. U.S. Department of Veterans Affairs (VA) provided funding to Robert A. Bonomo under grant number 1I01BX001974. School of Medicine, Case Western Reserve University (Case Western Reserve University School of Medicine) provided funding to Marisa L. Winkler under grant number T32 GM07250.

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