Biochemical Activity of Vaborbactam

Ruslan Tsivkovski and Olga Lomovskaya

Qpex Biopharma, Inc.
San Diego, CA, USA
olomovskaya@qpexbio.com

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Abstract

The most common mechanism of resistance to β-lactams antibiotics in Gram-negative bacteria is production of β-lactamase enzymes capable of cleaving the β-lactam ring. Inhibition of β-lactamase activity with small molecule drugs is a proven strategy to restore the potency of many β-lactam antibiotics. Vaborbactam (formerly RPX7009) is a cyclic boronic acid β-lactamase inhibitor (BLI) with a broad spectrum of activity against various serine β-lactamases, including KPC carbapenemases. The combination of vaborbactam and meropenem is approved in the US and Europe for the treatment of various nosocomial infections. We attempted to gain more insight into the mechanism of action of vaborbactam by conducting detailed kinetic characterization of its interaction with various recombinant His-tagged β-lactamases.

Vaborbactam demonstrated potent inhibition of class A and class C enzymes with $K_i$ values ranging from 0.022 μM to 0.18 μM, while inhibition of class D enzymes was rather poor and no activity against class B β-lactamases was detected. Importantly, vaborbactam inhibited KPC-2, KPC-3, BKC-1 and SME-2 carbapenemases at 1:1 stoichiometry, while these numbers were higher for other class A and C enzymes. Vaborbactam was also shown to be a potent progressive inactivator of several enzymes including KPCs with inactivation constants $k_2/K$ in the range of $3.4 \times 10^3$ to $2.4 \times 10^4$ M$^{-1}$* s$^{-1}$. Finally, experiments on the recovery of enzyme activity demonstrated high stability of the vaborbactam-KPC complex with 0.000040 s$^{-1}$ $k_{off}$ values and corresponding residence time of seven hours, whereas release of vaborbactam bound to other serine β-lactamases was substantially faster. The biochemical characteristics of vaborbactam described in this study may facilitate further chemical optimization efforts to develop boronic BLIs with improved affinity and broader spectrum of inhibition.
Introduction

The most common mechanism of resistance to β-lactams antibiotics in Gram-negative bacteria is production of β-lactamase enzymes capable of cleaving the β-lactam ring, resulting in complete loss of antibacterial activity. This family of enzymes has demonstrated tremendous growth over the past two decades and currently is represented by several structural classes (1). Of greatest concern is the recently observed spread of carbapenemase enzymes that can hydrolyze carbapenem antibiotics and threatens their clinical usefulness. In clinical settings, carbapenem-resistant Enterobacteriaceae (CRE) infections are associated with high rates of morbidity and mortality worldwide due to limited treatment options (2).

Inhibition of β-lactamase activity with small molecule drugs is a proven strategy to restore the potency of many β-lactam antibiotics (3). The long-ago discovered β-lactamase inhibitors (BLI) clavulanic acid and tazobactam (Figure 1) are potent against various class A and class C enzymes, but lack activity against many clinically relevant carbapenemases. Additional medicinal chemistry efforts resulted in development of a new non-β-lactam-based BLI avibactam (Figure 1) possessing activity against numerous serine enzymes including KPC carbapenemases (4). It has been approved for clinical use in combination with ceftazidime to treat complicated urinary tract infections (cUTI), hospital-acquired and ventilator-associated bacterial pneumonia (HABP and VABP) and complicated intra-abdominal infections (cIAI) in combination with metronidazole (5). Several compounds based on the same structural core are now at various stages of preclinical or clinical development (6-8). However, resistance
development to avibactam both in vitro and in clinical settings due to target \( \text{bla}_{KPC} \) mutations has been reported in multiple instances (9-12).

As an alternative structural scaffold, boronic acids have undergone extensive evaluation as inhibitors of serine \( \beta \)-lactamases due to formation of a stable covalent bond between the boron moiety and the active site serine residue (13-15). For instance, boronic acid BLI S02030 (Figure 1) was demonstrated to target a wide variety of serine enzymes, including ADC-7, KPC-2, and SHV-1 with nanomolar potency (16, 17). Recently, our efforts to develop more efficient inhibitors of carbapenemases led to discovery of vaborbactam (formerly RPX7009, Figure 1) – a cyclic boronic acid BLI with a broad spectrum of activity against class A and C \( \beta \)-lactamases (18). The most prominent feature of vaborbactam is its ability to inhibit KPC enzymes and to potentiate the activity of various carbapenems in CRE strains carrying these \( \beta \)-lactamases (19, 20). The combination of vaborbactam and meropenem is approved in the US for the treatment of complicated urinary tract infections (cUTI) (21) and in Europe for cUTI, complicated intra-abdominal infection (cIAI) and HABP/VABP (22). Importantly, in vitro multistep resistance development studies with the meropenem-vaborbactam combination using numerous clinical isolates that harbored KPC failed to generate any target mutations in \( \text{bla}_{KPC} \) genes (23).

Additionally, there were no reports of resistance development due to mutations in \( \beta \)-lactamase genes after extensive use of the meropenem-vaborbactam combination in clinical settings.

The vaborbactam structure represents a promising chemical scaffold for further development of BLIs with improved properties and broader spectrum of activity, which requires a deep understanding of the structural and kinetic aspects of interaction of the BLI with its enzymatic targets. Crystal structures of vaborbactam with CTX-M-15 and AmpC \( \beta \)-lactamases revealed the spatial orientation of the inhibitor molecule in the active site as well as a set of amino acid
residues involved in interaction with the BLI (18). However, a detailed kinetic characterization of vaborbactam interaction with various β-lactamases has been missing so far. In this investigation we attempted to gain more insight into the mechanism of vaborbactam binding kinetics with various serine β-lactamases with a specific emphasis on carbapenemases.

**Results and Discussion**

K\textsubscript{app} values of vaborbactam inhibition of various recombinant His-tagged β-lactamases from classes A, B, C and D were measured using a procedure previously employed for boronic BLIs (13, 24) ([Table 1](#)). Vaborbactam demonstrated the ability to inhibit the majority of class A serine carbapenemases. The activity of KPC-2 and KPC-3 enzymes was inhibited with 0.056 ± 0.015 μM and 0.050 ± 0.016 μM K\textsubscript{app} values, respectively. Also, recently discovered BKC-1 and FRI-1 carbapenemases (25, 26) that share very low homology with other serine β-lactamases were inhibited by vaborbactam with 0.018 ± 0.002 μM and 0.17 ± 0.06 μM K\textsubscript{app} values, respectively. The K\textsubscript{app} value of inhibition of the SME-2 enzyme from *S. marcescens* was 0.042 ± 0.005 μM. In addition to being a potent inhibitor of class A cabapenemases, vaborbactam demonstrated inhibitory activity against several ESBL as well as AmpC enzymes with K\textsubscript{i} values varying from 0.021 μM to 1.04 μM ([Table 1](#)). Vaborbactam demonstrated relatively poor inhibition of class D β-lactamases OXA-48 and OXA-23 resulting with K\textsubscript{app} values of 14 ± 5 μM and 66 ± 11 μM, respectively. Similar low affinity to OXA enzymes was reported for various arylboronic acid derivatives (27, 28). Crystal structures of some of these BLIs with OXA-24/40 revealed covalent bond formation between the boron and catalytic serine residues while various side chains attached to the arylboronate moiety were not involved in any specific interactions with the
generally hydrophobic catalytic site of the enzyme (28). Perhaps the lack of such interaction in
the oavorbactam-OXA complex could explain its significantly lower affinity. Interestingly,
inhibition experiments with a series of arylcycloboronate BLIs revealed that one of them
containing a cyclohexyl side chain inhibited various OXA enzymes with IC$_{50}$ values of 0.22 μM
and lower (29), suggesting that the constrained cycloboronate scaffold may be more suitable for
binding in the active site of class D enzymes.

No inhibition of the class B metallo-enzymes NDM-1 and VIM-1 by vaborbactam was detected.
This result is not surprising given the absence of a serine residue in the active site to form a
covalent bond with the inhibitor. However, several cycloboronate compounds were reported to
inhibit NDM-1, VIM-2 and BclII enzymes with IC$_{50}$ values ranging from 0.002 to 1 μM (29, 30),
suggesting a different mode of binding in the active site. Co-crystallization studies indeed
demonstrated interaction of the boron-bound oxygen atoms with the Zn$\text{I}$ ion in the substrate
binding pocket, while the bicyclic benzoxaborinine ring creates hydrophobic interactions with
the conserved Trp and Phe residues (30).

Mechanism-based suicidal BLIs (clavulanic acid, tazobactam, sulbactam) function by acylating
the catalytic serine residue of the enzyme (31). The resulting covalent complex can be
hydrolyzed by a water molecule, similar to typical β-lactam substrates, that leads to release of an
intact β-lactamase and the open ring form of the BLI molecule; alternatively, this complex can
undergo structural rearrangements resulting in irreversible enzyme inactivation (32). The number
of BLI molecules required to inactivate one molecule of β-lactamase is known as the
stoichiometry of inactivation or partition ratio. Consequently, we determined the stoichiometry
of inhibition of various serine enzymes that demonstrated a reasonable level of inhibition by
vaborbactam (Table 2). KPC-2, KPC-3, BKC-1 and SME-2 were inhibited by vaborbactam at a
1:1 ratio, while CTX-M-15, FRI-1 and AmpC demonstrated 2:1, 8:1 and 16:1 stoichiometry of inhibition by vaborbactam, respectively. For SHV-12 and TEM-43 enzymes it was impossible to reach complete inhibition even at the highest (256:1) molar ratio. The >1 stoichiometry ratios are unlikely due to vaborbactam hydrolysis but rather because of the specific conditions of the experiment that require enzyme:BLI complex formation at 1 μM concentration of enzyme. Subsequent dilution of the reaction mixture to determine residual enzyme activity results in quick inhibitor dissociation due to high vaborbactam k_{off} rates for those enzymes (see below). Importantly, unlike many suicidal BLIs no degradation of vaborbactam was observed after the inhibitor was incubated with KPC-2 for 18 hours and subjected to subsequent LC/MS analysis (Figure S1).

Mechanism-based BLIs are characterized by a two-step kinetic reaction pathway of the inhibitor binding to the enzyme (31). When studied using the reporter substrate method, this is manifested by progressive enzyme activity inactivation shown in Figure 2 for tazobactam with KPC-2 (right panel). In contrast, early reported boronic BLIs (e.g. m-tolyboronic acid and 2-formylphenylboronic acid (33) showed a linear KPC-2 inactivation profile, indicating that equilibrium between enzyme and inhibitor is very quickly established (Figure S2). However, boronic acid inhibitor S02030 (Figure 1) that is structurally very similar to vaborbactam demonstrated kinetic behavior with KPC-2 and SHV-1 enzymes similar to tazobactam (17). This led us to hypothesize that vaborbactam may also exhibit progressive inactivation profiles typical of covalent irreversible or slow tight binding reversible inhibitors. Kinetics of KPC-2 inactivation by vaborbactam demonstrated a slow onset of inhibition and non-linear reaction profiles (Figure 2). Similar inactivation profiles were obtained for all other enzymes presented in Table 3. These results suggest that vaborbactam interaction with these β-lactamases follows a
two-step kinetic mechanism. The first step is the formation of a non-covalent complex $EI_1$ characterized by binding constant $K$. The second step is a covalent interaction between the catalytic Ser residue of the enzyme and the boron atom of vaborbactam to form the $EI^*$ complex. This second step is characterized by the first-order rate constant $k_2$. Independent determination of these values was impossible due to the linear relationship between $k_{obs}$ and vaborbactam concentration values up to the highest inhibitor concentration tested (Figure S3). The inability to separately determine $K$ and $k_2$ values has been reported previously for various BLIs and $\beta$-lactamases from different structural classes (17, 34, 35). Therefore, the second-order rate constant $k_2/K$ for the onset of inhibition was calculated. Vaborbactam demonstrated comparable $k_2/K$ values of $5.5 \pm 0.5 \times 10^3$ and $6.7 \pm 0.3 \times 10^3$ M$^{-1}$s$^{-1}$ of inactivation of the KPC-2 and KPC-3 enzymes, respectively (Table 3). FRI-1 and SME-2 were inactivated by vaborbactam with similar efficiency, while BKC-1, CTX-M-15 and AmpC demonstrated higher efficiency of inactivation by vaborbactam with $k_2/K$ ranging from $1.2 \times 10^4$ to $2.4 \times 10^4$ M$^{-1}$s$^{-1}$. Overall, $k_2/K$ inactivation constants demonstrated only a four-fold difference between the lowest and highest values and were consistent with the results reported for other boronic BLIs (17). Interestingly, vaborbactam showed linear inactivation profiles with the SHV-12 and TEM-43 enzymes, which is characteristic of “fast on – fast off” boronic BLIs (Figure S2). This precluded calculation of the corresponding $k_2/K$ values (data not shown). It is quite likely that interaction of vaborbactam with these enzymes proceeded through simple one step formation of a covalent complex between the catalytic serine residue and the boron atom of vaborbactam, which can be rapidly hydrolyzed by a water molecule to release intact vaborbactam.

There have been no reports on the ability of boronic BLIs to induce structural rearrangements in $\beta$-lactamases that would result in irreversible enzyme modification as was reported for various $\beta$-
lactam-like BLIs (31). In general, the chemical bond between the boron atom of the BLI and serine residues can be hydrolyzed by a water molecule, releasing intact inhibitor and enzyme.

Taken together, this suggests that inhibition of β-lactamases by vaborbactam could be completely reversible upon removal of the BLI. The recovery of enzyme activity after complete inhibition by vaborbactam was studied by the jump dilution method (35). Activity recovery profiles for some enzymes are presented in Figure 3. Unlike $k_2/K$ the inactivation constant, the calculated $k_{off}$ values demonstrated a much higher degree of variation from $0.000030 \text{ s}^{-1}$ for KPC-3 to $0.0052 \text{ s}^{-1}$ for AmpC (Table 3). When these numbers were converted to residence time of enzyme-BLI complex, it resulted in a value of $559 \pm 28 \text{ min}$ for KPC-3 versus $3.2 \pm 0.2$ minutes for AmpC. Thus, vaborbactam forms remarkably stable complexes with KPC-2 and KPC-3 enzymes, while the stability of its complex with AmpC is substantially weaker. The $k_{off}$ values for the SHV-12 and TEM-43 enzymes could not be determined by the jump dilution method due to inability to completely inhibit their activity by vaborbactam, even at a very high 256:1 molar ratio. Next, $K_d$ values were calculated using $k_2/K$ and $k_{off}$ kinetic parameters (Table 3). They ranged from $4.4 \text{ nM}$ for the KPC-3 enzyme and up to $509 \text{ nM}$ for FRI-1. Such a high degree of variation can be attributed to the wide range of $k_{off}$ values whereas the difference between $k_2/K$ was not as dramatic. Interestingly, for KPC-2 and KPC-3 enzymes $K_d$ values were almost 10-fold lower than the corresponding $K_{app}$ values (Table 1). The difference could be attributed to the fact that $K_{app}$ measurements were done after enzyme incubation with BLI for 10 min while $K_d$ values reflect enzyme affinity at equilibrium.

In summary, we demonstrated that vaborbactam is a potent inhibitor of various serine β-lactamases belonging to structural classes A and C, including several carbapenemases. Of particular importance is the inhibition of clinically relevant KPC carbapenemases, which
contribute very strongly to the world-wide spread of CRE infections (36). Additionally, three
structurally diverse carbapenemase enzymes SME-2, BKC-1 and FRI-1 are also inhibited by
vaborbactam with reasonable potency. Regarding the mechanism of inhibition, with the majority
of the tested enzymes, vaborbactam behaves as a slow tight binding inhibitor that requires two
distinct kinetic steps for enzyme inactivation. Crystal structures of vaborbactam complexes with
CTX-M-15 and AmpC β-lactamases demonstrated an extensive interaction network formed
between the amino acid residues surrounding the enzyme substrate binding pocket and the
carboxy and amide groups of the inhibitor molecule (18). One might speculate that this
interaction could possibly contribute to the biphasic kinetic behavior of vaborbactam with certain
enzymes. Another important finding in this study is almost irreversible binding of vaborbactam
to the KPC carbapenemases, with calculated residence times reaching several hours. Comparison
of molecular structures of vaborbactam complexes with KPC-2 (manuscript in preparation) vs
other enzymes may shed light on the structural elements responsible for this phenomenon. The
increased residence time of the BLI:enzyme complex may also have a positive effect on the in
vivo potency of vaborbactam in animal infection models (37). Overall, the biochemical
characteristics of vaborbactam described in this study will be useful for further chemical
optimization efforts to develop boronic BLIs with improved affinity and a broader spectrum of
inhibition.

Materials and Methods
Purification of KPC-2 and KPC-3 proteins for biochemical studies. Full KPC-2 and KPC-3 gene coding sequences were cloned into pET28a vector that produced an expression construct with periplasmic protein secretion and 6xHis-tag on its C-terminus. The recombinant plasmids were transformed into BL21(DE3) pLys strain. 2 mL of overnight culture was inoculated in 1L of LB media with 50 μg/mL of kanamycin and 20 μg/mL of chloramphenicol and grown at 37°C with 300RPM shaking until reaching OD₆₀₀=0.7-0.8. IPTG was added to 0.2 mM concentration and cells continued to grow for additional 3h. Cells were harvested by centrifugation and the pellet was resuspended in 40 mL of ice-cold 50 mM TrisHCl pH 8.0, 500 mM sucrose, 1 mM EDTA and 1 tablet of complete protease inhibitor (Roche-Sigma-Aldrich, St. Louis, MO USA). The suspension was incubated on ice with six cycles of 15 sec vortexing with five minutes pause in-between. The suspension was centrifuged 30 min at 30000xg, the supernatant collected, then sonicated for 30 sec to reduce viscosity and MgCl₂ and imidazole were added to 2 mM and 5 mM concentrations, respectively. The lysate was loaded by gravity flow onto a 1mL column with HisPur Cobalt Resin (Thermo Scientific, USA) pre-equilibrated with 50 mM Na-phosphate pH7.4, 300 mM NaCl, 5 mM imidazole buffer. The column was washed with 40 mL of the same buffer and consequently His-tag protein was eluted with 50 mM Na-phosphate pH7.4, 300 mM NaCl, 70 mM imidazole buffer. All wash and elution fractions were analyzed by 8-16% SDS-PAGE. Fractions containing target protein were pooled, concentrated and dialyzed against 50 mM Na-phosphate pH 7.0. Purity of all proteins were at least 95% as determined by SDS-PAGE. Protein preparations were aliquoted and stored at -20°C until further use.

Purification of OXA-23, BKC-1, FRI-1 and SME-2 proteins for biochemical studies. Coding sequences for all four proteins were cloned into a pET28a vector that produced an expression construct with cytoplasmic protein localization and N-terminal 6xHis-tag. The recombinant
plasmids were transformed into BL21(DE3) strain. 25 mL of overnight culture grown in LB at 30°C was inoculated in 1L of MagicMedia™ (ThermoFisher Scientific, USA) with 25 µg/mL of kanamycin and grown at 18°C with 300RPM shaking for 32h. Cells were harvested by centrifugation and the pellet was resuspended in 40 mL of ice-cold 50 mM Na-phosphate pH 7.5, 300 mM NaCl, 1 tablet of complete protease inhibitor tablet. The suspension was subjected to six cycles of one min sonication with five minutes pause in-between on ice. The suspension was centrifuged 1h at 30000xg, the supernatant was collected, and imidazole was added to 5 mM concentrations. Further enzyme purification was performed using the same approach as with KPC-2 and KPC-3.

All other enzymes used in the study were expressed and purified by Emerald Biostructures (Bainbridge Island, WA, USA).
Determination of vaborbactam $K_i$ values for various $\beta$-lactamases. Protein was mixed with various concentrations of inhibitors in 50 mM Na-phosphate pH7.0, 0.1mg/ml of BSA buffer (reaction buffer) and incubated for 10 min at 37°C. 50 $\mu$M NCF (10 $\mu$M for SHV-12 and 25 $\mu$M for BKC-1) was added and substrate cleavage profiles were recorded at 490 nm every 10 sec for 10 min. NCF concentrations for $K_i$ determinations were selected not to exceed $K_m$ values by more than 2-fold to prevent “saturation” of enzyme activity with substrate. $K_{app}$ values were calculated by method of Waley SG (24).

Stoichiometry of $\beta$-lactamase inhibition by vaborbactam. Enzyme at 1 $\mu$M in reaction buffer was mixed with BLI at molar ratios varying from 256 to 0.0625. After 30 min incubation at 37°C, the reaction mixture was diluted 200-fold and enzyme activity was measured with NCF as described above. Stoichiometry of inhibition was determined as minimal BLI:enzyme ratio reducing enzyme activity to less than 10%.

Determination of vaborbactam $k_2/K$ inactivation constant for various $\beta$-lactamases. Inactivation kinetic parameters were determined by reporter substrate method for slow tight binding inhibitor kinetic scheme (38).

\[
\begin{align*}
K & \quad k_2 \\
E + I & \leftrightarrow EI & \leftrightarrow EI^* \\
& \quad k_2 \\
\end{align*}
\]

Protein was quickly mixed with 100 $\mu$M nitrocefin and various concentrations of BLI in reaction buffer and absorbance at 490 nm was measured immediately every two seconds for 180 sec on SpectraMax plate reader (Molecular Devices, San Jose, CA, USA) at 37°C. Resulting progression curves of OD$_{490}$ vs time at various BLI concentrations were imported into Prism.
software (GraphPad, San Diego, CA, USA) and pseudo first-order rate constants $k_{\text{obs}}$ were calculated using the following equation

$$P = V_s \cdot (1 - e^{-k_{\text{obs}} t}) / k_{\text{obs}}$$

where $V_s$ - enzyme NCF cleavage rate in the absence of BLI. $k_{\text{obs}}$ values calculated at various vaborbactam concentrations were fitted in the following equation

$$k_{\text{obs}} = k_2 + k_2 / K \cdot [I] / (1 + [NCF] / K_m(\text{NCF}))$$

where

- $k_2 / K$ - inactivation constant
- $[I]$ – inhibitor concentration
- $[NCF]$ – nitrocefin concentration
- $K_m(\text{NCF})$ - Michaelis constant of NCF for $\beta$-lactamase

The following enzyme NCF $K_m$ values were used for $k_2 / K$ calculation: KPC-2 - 49±5 μM, KPC-3 - 49±2 μM, BKC-1 – 9.4±1.4 μM, FRI-1 - 75±4 μM, SME-2 - 59±3 μM, CTX-M-15 - 24±3 μM, AmpC - 33±3 μM.
Determination of $k_{\text{off}}$ rates of enzyme activity recovery after inhibition by vaborbactam. Purified enzyme at 1 μM concentration in reaction buffer was mixed with BLIs at 8-fold higher concentration than its stoichiometry ratio (determined in preliminary stoichiometry experiments). After 30 min incubation at 37°C, the reaction mixture was diluted from 100-fold to 30000-fold depending on the enzyme in reaction buffer and 100 μL of diluted enzyme was mixed with 100 μL of 400 μM NCF in reaction buffer. Absorbance at 490 nm was recorded every minute during 4h at 37°C. Resulting reaction profiles were fitted into the following equation using Graph Pad Prizm software to obtain $k_{\text{off}}$ values: $P=V_s*t+(V_o-V_s)*(1-e^{-k_{\text{off}}t})/k_{\text{off}}$, where $V_s$ – uninhibited enzyme velocity, measured in the reaction with enzyme and no inhibitor, $V_o$ – completely inhibited enzyme velocity, measured in the reaction with no enzyme and NCF only.

**Statistical analysis.** All kinetic results are presented as average ± standard deviation of minimum three replicates.

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Table 1. $K_{iapp}$ values of vaborbactam inhibition of various $\beta$-lactamases

| Enzyme   | Class | Carbapenemase | $K_{iapp} (\mu M)$ |
|----------|-------|---------------|-------------------|
| KPC-2    | A     | +             | 0.056 ± 0.015     |
| KPC-3    | A     | +             | 0.050 ± 0.016     |
| BKc-1    | A     | +             | 0.018 ± 0.002     |
| FRI-1    | A     | +             | 0.17 ± 0.06       |
| SME-2    | A     | +             | 0.042 ± 0.005     |
| CTX-M-14 | A     | -             | 0.033 ± 0.013     |
| CTX-M-15 | A     | -             | 0.030 ± 0.004     |
| SHV-12   | A     | -             | 0.021 ± 0.004     |
| TEM-10   | A     | -             | 0.14 ± 0.04       |
| TEM-43   | A     | -             | 1.04 ± 0.20       |
Table 2. Stoichiometry* of vaborbactam inhibition of various β-lactamase enzymes

| Enzyme      | Stoichiometry | \( K_{IC50} \) (M* s\(^{-1}\)) | \( k_{off} \), s\(^{-1}\) | Residence time, min | \( K_d \), nM |
|-------------|---------------|----------------------------------|-----------------------------|---------------------|-----------|
| KPC-2       | 1             | 5.5 ± 0.5 \times 10^1            | 0.000043 ± 0.000006         | 394 ± 50            | 7.8       |
| KPC-3       | 1             | 6.7 ± 0.3 \times 10^1            | 0.000030 ± 0.000001         | 559 ± 28            | 4.4       |
| BKC-1       | 1             | 1.2 ± 0.1 \times 10^1            | 0.00040 ± 0.00008           | 43 ± 8              | 33        |
| FRI-1       | 8             | 3.4 ± 0.1 \times 10^1            | 0.0017 ± 0.0001             | 9.8 ± 0.7           | 509       |
| SME-2       | 1             | 5.0 ± 0.2 \times 10^1            | 0.00024 ± 0.00002           | 71 ± 7              | 47        |
| CTX-M-15    | 2             | 2.3 ± 0.2 \times 10^1            | 0.0009 ± 0.0002             | 19 ± 1              | 40        |

* number of inhibitor molecules required to reduce enzyme activity by less than 10%

Table 3. Kinetic parameters of vaborbactam inactivation of various β-lactamases

| Enzyme      | \( k_2/K \) (M\(^{-1}\) * s\(^{-1}\)) | \( k_{off} \), s\(^{-1}\) | Residence time, min | \( K_d \), nM |
|-------------|--------------------------------------|-----------------------------|---------------------|-----------|
| KPC-2       | 5.5 ± 0.5 \times 10^1                | 0.000043 ± 0.000006         | 394 ± 50            | 7.8       |
| KPC-3       | 6.7 ± 0.3 \times 10^1                | 0.000030 ± 0.000001         | 559 ± 28            | 4.4       |
| BKC-1       | 1.2 ± 0.1 \times 10^1                | 0.00040 ± 0.00008           | 43 ± 8              | 33        |
| FRI-1       | 3.4 ± 0.1 \times 10^1                | 0.0017 ± 0.0001             | 9.8 ± 0.7           | 509       |
| SME-2       | 5.0 ± 0.2 \times 10^1                | 0.00024 ± 0.00002           | 71 ± 7              | 47        |
| CTX-M-15    | 2.3 ± 0.2 \times 10^1                | 0.0009 ± 0.0002             | 19 ± 1              | 40        |
Figure 1. Chemical structures of various BLIs

Figure 2. Kinetic profiles of KPC-2 inactivation by vaborbactam and tazobactam. Vaborbactam and tazobactam at indicated concentration (in μM) were quickly mixed with 1.2 nM KPC-2 enzyme and 100 μM NCF as reporter substrate and absorbance at 490 nm was recorded immediately every 2 sec using plate reader.
Figure 3. Kinetic profiles of activity recovery of various β-lactamases after inhibition by vaborbactam using jump dilution method. Enzymes at 1 μM concentration were mixed with vaborbactam at concentration 8-fold higher than stoichiometry ratio and incubated for 30 min. After appropriate dilution 100 μM NCF was added to the reaction mixture and absorbance at 490 nm was recorded every 10 sec using plate reader. Reaction without addition of BLI was also recorded and used to calculate uninhibited enzyme velocity \( V_\text{s} \).