Biochemical Characterization of QPX7728, a New Ultra-Broad-Spectrum Beta-lactamase Inhibitor of Serine and Metallo-Beta-Lactamases

Short title: Biochemical characterization of QPX7728

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QPX7728 is a new ultra-broad-spectrum inhibitor of serine and metallo beta-lactamases from a class of cyclic boronates that gave rise to vaborbactam. The spectrum and mechanism of beta-lactamase inhibition by QPX7728 were assessed using purified enzymes from all molecular classes. QPX7728 inhibits class A ESBLs (IC$_{50}$ range 1-3 nM) and carbapenemases such as KPC (IC$_{50}$ 2.9±0.4 nM) as well as class C P99 (IC$_{50}$ of 22±8 nM) with a potency that is comparable or higher than recently FDA approved BLIs avibactam, relebactam and vaborbactam. Unlike those other BLIs, QPX7728 is also a potent inhibitor of class D carbapenemases such as OXA-48 from Enterobacteriaceae and OXA enzymes from A. baumannii (OXA-23/24/58, IC$_{50}$ range 1-2 nM) as well as MBLs such as NDM-1 (IC$_{50}$ 55±25 nM), VIM-1 (IC$_{50}$ 14±4 nM) and IMP-1 (IC$_{50}$ 610±70 nM). Inhibition of serine enzymes by QPX7728 is associated with progressive inactivation with a high efficiency k$_2$/K ranging from 6.3 x 10$^4$ (for P99) to 9.9 x 10$^5$ M$^{-1}$ s$^{-1}$ (for OXA-23). This inhibition is reversible with variable stability of the QPX7728-beta-lactamase complexes with target residence time ranging from minutes to several hours: 5-20 minutes for OXA carbapenemases from A. baumannii, ~50 minutes for OXA-48 and 2-3 hours for KPC and CTX-M-15. QPX7728 inhibited all tested serine enzymes at 1:1 molar ratio. Metallo-beta-lactamases NDM, VIM, and IMP were inhibited by a competitive mechanism with fast-on-fast-off kinetics, with Ks of 7.5±2.1 nM, 32±14 nM and 240±30 nM for VIM-1, NDM-1 and IMP-1, respectively. QPX7728 ultra-broad-spectrum of BLI inhibition combined with its high potency enables combinations with multiple different beta-lactam antibiotics.

Key words: QPX7728, beta-lactamase inhibition kinetics, serine beta-lactamases, metallo-beta-lactamases
INTRODUCTION

Beta-lactam antibiotics belonging to four major classes: penicillins, cephalosporins, monobactams and carbapenems; collectively, they are the most widely-used group of antimicrobial agents for treatment of bacterial infections, in both community (oral penicillins and oral cephalosporins) and hospital settings (penicillins, cephalosporins, monobactams and carbapenems) owing to their broad-spectrum nature, bactericidal mode of action, and excellent safety profile (1, 2). However, resistance to all types of beta-lactams has emerged in the US (3) and worldwide (4), limiting the utility of this important class of antibiotics. The most significant mechanism that mediates resistance to beta-lactams is the production of beta-lactamases, inactivating enzymes that hydrolyze the amide bond of the beta-lactam ring (5).

These diverse beta-lactamases are grouped into four molecular classes based on sequence and structural similarity (6, 7). Beta-lactamases that belong to classes A, C and D are the so-called serine enzymes (SBLs); the nucleophilic serine in their active center is required for the hydrolytic reaction. Beta-lactamases from Class B require Zn(II) for their activity and thus are metallo-enzymes or MBLs. MBLs are divided into three sub-classes, B1, B2, and B3, based on sequence similarity (8). Beta-lactamases from different molecular classes often have overlapping substrate specificity (7).

Development of beta-lactamase inhibitors (BLIs) in combination with beta-lactams is a powerful strategy to protect beta-lactams from beta-lactamase-mediated hydrolysis, thus extending their clinical utility. The first three BLIs that reached the clinic in the 80s and 90s, clavulanic acid, tazobactam and sulbactam, are all beta-lactam-based molecules that are irreversible suicidal inhibitors. They inactivate beta-lactamases in a stepwise process that involves the opening of the beta-lactam ring of the BLIs and its irreversible covalent binding to the active site serine. These BLIs have a limited spectrum that is restricted mainly to class A penicillinas (TEM-1, SHV-1) and ESBLs (extended spectrum beta-lactamases) from SHV, TEM, CTX-M sub-families (9). They do not inhibit the class A carbapenemase KPC because KPC can efficiently hydrolyze these beta-lactam-type molecules in the same way it does beta-lactam antibiotics (10).
Three newer BLIs that are now in clinical use, avibactam (approved by the FDA in 2015 in combination with ceftazidime), vaborbactam (approved by the FDA in 2017 in combination with meropenem) and relebactam (approved by the FDA in 2019 in combination with imipenem), are all derived from non-beta-lactam scaffolds (Figure 1) (11). Avibactam and relebactam are diazabicyclooctane derivatives (DBOs) while vaborbactam is based on a cyclic boronic acid pharmacophore (12). These three BLIs are dual inhibitors of class A and class C beta-lactamases and all three are potent inhibitors of KPC. Avibactam (but not relebactam or vaborbactam) can also inhibit some class D enzymes (13). Similar to clavulanic acid and tazobactam, beta-lactamase inhibition by avibactam (and other DBOs) involves covalent binding of the opened ring inhibitor to the active site serine (14, 15).

However, unlike beta-lactam-based BLIs, this binding is slowly reversible due to recyclization of avibactam which in most cases is followed by the release of intact enzyme and intact avibactam. The exception is KPC which can slowly desulfate the avibactam adduct resulting in slow inactivation of avibactam (14) [and other DBOs, (16)]. As this inactivation is very slow, it apparently does not affect antibiotic potentiation activity of avibactam and other DBOs.

The mechanism of inhibition of beta-lactamases by vaborbactam is also based on a reversible covalent binding where the boronate of vaborbactam makes a covalent bond with the active site serine forming a tetrahedral intermediate and functioning as a transition state analog (12). This process does not involve the opening of the vaborbactam ring; the intact compound is released from the enzyme. An appealing feature of vaborbactam is an unusually slow dissociation rate of the KPC-vaborbactam complex with a target residence time of several hours (17). No degradation of vaborbactam by KPC has been detected (17).

Combination agents recently approved by the FDA based on the three BLIs described above represent significant progress in addressing serious drug-resistant gram-negative infections, particularly those caused by KPC-producing carbapenem-resistant Enterobacteriaceae; however, none of these BLIs inhibit metallo beta-lactamases, hence, the combination agents not active against metallo beta-lactamase producing CRE (carbapenem resistant Enterobacteriaceae). Likewise, since none of these BLIs inhibit...
class D carbapenemases in *Acinetobacter baumanii*, these agents do not have utility against this important pathogen (18). Of note, none of these agents have oral formulations available for the treatment of infections due to serious or urgent resistant threats in settings where IV therapy is not available or desirable (19).

Prompted by this ongoing unmet clinical need, we initiated a program that involved modifications to the boronic acid pharmacophore with a goal of expanding the spectrum of beta-lactamase inhibition to include both serine and metallo beta-lactamases and to achieve oral bioavailability. QPX7728 was discovered that has properties that achieved our preclinical targets in a single molecule. QPX7728 is an ultra-broad-spectrum inhibitor with activity against numerous serine and metallo beta-lactamases, including carbapenemases such as class A KPC, class B NDM and VIM, and class D OXA-48 and OXA-23 that are found in carbapenem-resistant *Enterobacteriaceae* and in carbapenem-resistant *Acinetobacter baumanii*, respectively. In addition, QPX7728 can be delivered by oral administration (20); thus, the ultra-broad-spectrum beta-lactamase inhibition spectrum could be applied to IV and oral QPX7728-based combination products.

**RESULTS AND DISCUSSION**

QPX7728 is an ultra-broad-spectrum inhibitor of diverse serine and metallo-beta-lactamases.

The inhibition profile of QPX7728 (Figure 1) (defined as IC$_{50}$) of inhibition of hydrolysis of nitrocefin or imipenem (for NDM and IMP MBLs) by several purified beta-lactamases from all four molecular classes is shown in Table 1. QPX7728 inhibited class A ESBLs, CTX-M-14 and CTX-M-15, SHV-12, TEM-10 and TEM-26 with IC$_{50}$ values in a 1-3 nM range, a potency generally similar to avibactam and greater than relebactam or vaborbactam. QPX7728 was also a potent inhibitor of KPC-2 with an IC$_{50}$ of ~3 nM. IC$_{50}$ values of KPC-2 inhibition for the comparator BLIs vaborbactam, avibactam and relebactam ranged from 22 nM to 110 nM (Table 1). The IC$_{50}$ for inhibition of class C beta-lactamase P99 from *E. cloacae* by QPX7728 was ~22 nM and was similar to that for comparator BLIs. QPX7728 also demonstrated potent inhibition of class D carbapenemase OXA-48 with an IC$_{50}$ of 1 nM.
which is 160-fold more potent than avibactam. As reported earlier, neither relebactam (21) nor
vaborbactam (17) inhibit OXA-48. Importantly, the same high potency (IC$_{50}$ of 1 nM), was observed for
QPX7728 inhibition of OXA-23, the class D carbapenemase from *A. baumannii*. None of the comparator
BLIs had activity against this enzyme (Table 1). For vaborbactam, which relies on the favorable
interactions of its amide ‘side-chain’ carbonyl (Figure 1) for some of its potency (12), the reason for its
lack of inhibition of OXA enzymes seems to be related to availability of hydrogen bond donors. In class
A enzymes there are hydrogen bond donors that interact with the amide carbonyl, while OXA enzymes
lack these donors. Instead the corresponding region is hydrophobic and quite enclosed in class D (22). For
vaborbactam, this results in unfavorable contacts, diminishing activity against OXA enzymes. QPX7728
lacks this amide and apparently avoids this problem with a resulting increase in potency towards OXA
enzymes.

Notably, QPX7728 inhibited clinically important MBLs with considerable potency. VIM-1 and
NDM-1 were inhibited with IC$_{50}$ values ranging from 14 nM to 55 nM. Somewhat lower potency was
observed for MBLs from the IMP sub-group, 0.61 µM and 4 µM for IMP-1 and IMP-26, respectively
(Table 1). As expected, none of the comparator BLIs had any MBL inhibitory activity (IC$_{50}$ > 160 µM).
MBLs that are inhibited by QPX7728 belong to the B1 sub-class of the class B family; this sub-
class contains the most clinically relevant beta-lactamases, such as NDM and VIM (8). Two other sub-
classes, B2 and B3, that represent less common enzymes, differ from B1 based on sequence and structure
(in particularly, B3), amino acid residues involved in Zn coordination and even the number of Zn atoms
involved in catalysis. No QPX7728 inhibition experiments have been performed with purified B2 and B3
MBLs up to date. However, microbiological experiments demonstrated the inability of QPX7728 to
reduce MIC of meropenem and ceftazidime against the strain producing cloned L1 from
*Stenotrophomonas maltophilia*, the MBL from the B3 subclass (23), suggesting that QPX7728 is a poor
inhibitor of L1, . This suggests that a different structural scaffold may be required for interaction with this
group of MBLs.
QPX7728 is a covalent slowly reversible inhibitor of serine beta-lactamases

The kinetic mechanism of QPX7728 interaction with serine and metallo-beta-lactamases was studied using nitrocefin and several purified enzymes (kinetic parameters for all the beta-lactamases used in these studies are presented in the Supplementary Table S1). The profiles of inactivation of beta-lactamase activity by QPX7728 for selected enzymes are presented in Figure 2a. For all the tested serine beta-lactamases, QPX7728 demonstrated biphasic inactivation kinetics with a slow onset of inhibition and non-linear reaction profiles characteristic of progressive inactivation of activity. Additionally, this kinetic behavior is manifested by the decrease of apparent $K_i$ values ($K_i$ app) when enzyme and BLI are pre-incubated for various times (Supplementary Figure S1). This type of kinetic behavior is manifested by the decrease of apparent $K_i$ values ($K_i$ app) when enzyme and BLI are pre-incubated for various times (Supplementary Figure S1). This type of kinetics is described by the initial formation of a non-covalent complex EI (characterized by binding constant $K$) that subsequently proceeds to a covalent interaction between the catalytic serine residue of the enzyme and a boron atom of a BLI in the EI* complex (24). This second step is described by the first-order rate constant $k_2$ (Equation 1).

$$K \begin{array}{c} k_2 \\ \rightarrow \end{array} E + I \leftrightarrow EI \leftrightarrow EI^* \quad \text{Equation 1}$$

Enzyme inactivation efficiency is defined by the second order constant $k_2/K$ which was determined for several class A, class C and class D beta-lactamases (Table 2). QPX7728 efficiently inactivated all tested beta-lactamases: the lowest $k_2/K$ was for the Class C P99 enzyme and was still of an appreciable value of $\sim 6 \times 10^4$ M$^{-1}$ s$^{-1}$. For class A beta-lactamases, the inactivation constant $k_2/K$ of QPX7728 varied over a 10-fold range with the lowest and the highest inactivation efficiency of $1.0 \times 10^5$ M$^{-1}$ s$^{-1}$ and $1.8 \times 10^6$ M$^{-1}$ s$^{-1}$ for SHV-12 and BKC-1, respectively. $k_2/K$ for KPC-2 and KPC-3 were $3.4 \times 10^5$ M$^{-1}$ s$^{-1}$ which is almost 100-fold higher compared to vaborbactam. QPX7728 inhibited all tested class D carbapenemases with a high efficiency: $k_2/K$ for OXA-48 from Enterobacteriaceae was $\sim 3 \times 10^6$ M$^{-1}$ s$^{-1}$ and $k_2/K$ for OXA carbapenemases from A. baumannii (OXA-23, OXA-24, OXA-58) was $\sim 1 \times 10^6$ M$^{-1}$ s$^{-1}$.

The crystal structures of several serine beta-lactamases, CTX-M-14, KPC-2, and OXA-48 bound to QPX7728 were recently determined (20). In these enzymes, the catalytic serine residue is covalently
bound to the boron atom of the inhibitor validating applicability of the kinetic model that was used to
determine the inactivation efficiency of QPX7728.

The reversibility of inhibition of serine beta-lactamases by QPX7728 was evaluated by the “jump
dilution” method as previously described for avibactam (15) and vaborbactam (17). QPX7728 produced
typical enzyme recovery profiles characteristic of reversible inhibition (Figure 3 for some of the recovery
curves). $k_{\text{off}}$ values for different beta-lactamases varied significantly (Table 2). For Class A beta-
lactamases they ranged from 0.003 s$^{-1}$ for SHV-12 to 0.00008 s$^{-1}$ for CTX-M-15. That translates to
QPX7728 residence time values of 5 minutes and 220 minutes, respectively. The residence time for KPC-
2/KPC-3 was similar to that of CTX-M-15, 130-190 minutes. A low $k_{\text{off}}$ value of 0.000033 s$^{-1}$ with a
residence time of ~500 minutes was determined for P99 inhibition by QPX7728. Finally, among class D
carbapenemases the lowest $k_{\text{off}}$ values of ~0.0004 s$^{-1}$ and the longest residence time of 47 minutes was
observed for OXA-48. $k_{\text{off}}$ and residence time values for OXA carbapenemases from A. baumannii were
in the 0.001-0.004 s$^{-1}$ range and 5-20 minutes range, respectively.

QPX7728 had 3-fold to 150-fold lower $k_{\text{off}}$ values and longer residence time with most enzymes
compared to vaborbactam. The only exceptions were KPC-2 and KPC-3 (17) for which vaborbactam
demonstrated more stable complex formation than QPX7728 with 2-3-fold lower $k_{\text{off}}$ values. The detailed
analysis of the recently solved structures of vaborbactam and QPX7728 bound to KPC is in progress and
might provide the structural basis for the observed differences.

Nevertheless, given ca. 100-fold higher inactivation efficiency of KPC by QPX7728 compared to
vaborbactam, QPX7728 $K_d$ values for KPC-2 and KPC-3 were in a sub-nanomolar range that was 10-50-
fold lower compared to that of vaborbactam (Table 2, (17)).

Overall, QPX7728 $K_d$ values for various beta-lactamases ranged from 0.06 nM to 28 nM and
from 0.13 nM to 3.2 nM for class A and for class D enzymes, respectively. $K_d$ for P99 was 0.59 nM
(Table 2). QPX7728 inhibited all tested serine enzymes at 1:1 molar ratio (Table 2).

QPX7728 is a “fast-on-fast-off” inhibitor of MBLs
A very different kinetic inhibition profile by QPX7728 was observed for the metallo-beta-lactamases NDM-1, VIM-1 and IMP-1 (Figure 2b): QPX7728 produced linear inactivation profiles, with no sign of progressive inactivation indicative of the quickly established equilibrium between the enzyme and an inhibitor which is typical for “fast on - fast off” reversible inhibitors. Also, no differences in the QPX7728 $K_{\text{app}}$ values were observed with variation in the pre-incubation times of QPX7728 with these MBLs (Supplementary Figure S1), contrary to KPC-2 enzyme supporting their reversible inhibition by QPX7728. Fast and reversible inhibition is consistent with a non-covalent complex that QPX7728 forms with NDM-1 and VIM-1 based on available structural information (20).

Further evaluation revealed that QPX7728 behaved as a competitive inhibitor of MBLs (Figure 4) with $K_s$ of 7.5 nM, 32 nM and 240 nM, for VIM-1, NDM-1 and IMP-1, respectively. Given the “fast on - fast off” reversible inhibition mechanism these $K_s$ can be considered as an equivalent of $K_d$ values of QPX7728 for MBLs (Table 2).

QPX7728 has the broadest spectrum of beta-lactamase inhibition compared to the marketed BLIs avibactam, relebactam and vaborbactam. These marketed agents have activity against class A beta-lactamases including carbapenemases such as KPC, class C beta-lactamases and some class D enzymes (in the case of avibactam). QPX7728 has two main improvements in spectrum: it is an efficient inhibitor of class D carbapenemases from A. baumannii such as OXA-23, OXA-24/40, OXA-58 and it inhibits various class B metallo-beta-lactamases from the B1 sub-class such as NDM, VIM, IMP and others (but not the B3 sub-class such as L1 from S. maltophilia).

Two other investigational BLIs, the avibactam derivative DBO durlobactam (ETX2514) and the bicyclic boronate taniboractam (VNRX-5133) are in clinical trials (11). Durlobactam is a potent inhibitor of class A, class C and class D beta-lactamases including those from A. baumannii (25, 26).

Based on comparing with published data (16, 25, 26), QPX7728 and durlobactam have similar $K_s$ for OXA carbapenemases from A. baumanii, which is in the low nanomolar range. But unlike QPX7728, durlobactam does not inhibit metallo beta-lactamases.
Taniborbactam inhibits both serine and metallo-beta-lactamases. Based on published data, QPX7728 and taniborbactam appear to have similar potency of inhibition of serine beta-lactamases, and MBLs, NDM and VIM. In contrast, QPX7728 is a more potent IMP inhibitor compared to taniborbactam. Unlike QPX7728, taniborbactam, does not inhibit class D carbapenemases from A. baumannii (27-29).

We speculate that the exceptional breadth of inhibition spectrum of QPX7728 is likely, at least in part, due to its very compact structure – the inhibitor is barely larger than the 5/4 core ring system of the beta lactam substrates (Figure 5) and fits entirely within the immediate vicinity of the active site. Lack of any side-chains extending into more distal regions allows the inhibitor to avoid potentially unfavorable interactions that could arise due to sequence/structure variations. Notably, the expansion of spectrum achieved in QPX7728 did not come at the expense of reduced potency against the class A and class C beta-lactamases; for example, QPX7728 has ca. 8-30-fold higher potency against KPC-2 compared to avibactam, relebactam and vaborbactam and appears to have similar potency against class C P99.

We speculate that one significant factor enhancing the potency of QPX7728 compared to vaborbactam is its near-total rigidity. Thus, conformational entropy loss upon binding is negligible for QPX7728. In contrast, vaborbactam has 5 rotatable bonds and, as evidenced by X-ray structures (12), a ring with two alternative low energy conformations, a total of 6 degrees of freedom. The entropic penalty upon binding for each lost degree of conformational freedom might result in a significantly increased $K_d$; a ballpark estimate of 0.6kcal/mol per degree of freedom would give a 3.6kcal/mol free energy penalty, equivalent to a several hundred-fold difference in $K_d$.

QPX7728 demonstrates progressive inactivation of all serine beta-lactamases and its inactivation efficiency is generally very high. For example, QPX7728 inactivates KPC-2 ($k_2/K$ of $3.6 \times 10^9$ M$^{-1}$ s$^{-1}$) ca. 10-fold more efficiently than avibactam ($k_2/K$ of $1.3 \times 10^8$ (14)) and relebactam ($k_2/K$ of $2.5 \times 10^8$ (30)) and almost 100-fold more efficiently than vaborbactam ($k_2/K$ of $5.5 \times 10^7$ (17)). In addition to the high inactivation efficiency, QPX7728 forms a very tight complex with some serine beta-lactamases with calculated residence times reaching several hours. In the case of KPC-2, the residence time of QPX7728 is 3 hours. This is comparable to ~2 hours for avibactam (14), 1.5 hours for relebactam (30) and ~6 hours.
for vaborbactam (17). Thus, QPX7728 is a covalent low off-rate inhibitor of serine enzymes. In contrast, inhibition of metallo-beta-lactamases by QPX7728 [similar to taniborbactam (27)] proceeds through a simple one-step complex formation typical for “fast on -fast off” inhibitors.

CONCLUSIONS

QPX7728 has an ultra-broad spectrum of beta-lactamase inhibition. It has two major improvements in spectrum compared to the currently marketed agents (avibactam, vaborbactam, and relebactam) and clinical stage investigational agents (durlobactam and taniborbactam): it is an efficient inhibitor of class D carbapenemases from A. baumannii such as OXA-23, OXA-24/40, OXA-58, and it inhibits various class B metallo-beta-lactamases from the B1 sub-class such as NDM, VIM, and IMP.

Methods

Beta-lactamase enzyme preparations. All purified beta-lactamase enzymes used in the study were either expressed and purified internally (17) or obtained from Emerald Biostructures (Bainbridge Island, WA).

Determination of IC\textsubscript{50} and K\textsubscript{i} values of beta-lactamase inhibition by BLI with nitrocefin or imipenem as a substrate. Enzymes were mixed with BLIs at concentrations varying from 160 to 0.0027 µM in 50 mM Na-phosphate pH7.0, 0.1 mg/ml BSA (buffer A, 20 µM ZnCl\textsubscript{2} was also added for all metallo enzymes) and incubated for 10 minutes at 37°C. 50 µM nitrocefin (10 µM for SHV-12) or 100 µM of imipenem (pre-warmed at 37°C for 10 minutes) was added and substrate cleavage profiles were recorded at 37°C at 490 nm every 10 seconds for 10 minutes or at 294 nm every 30 seconds for 1 hour for nitrocefin and imipenem, respectively. Initial rates of reaction were calculated and exported to Prizm software to calculate IC\textsubscript{50} values using “dose-response – inhibition, variable slope (four
parameters)” equation. $K_i$ values were calculated by method of Waley SG (31). This method was previously used to calculate $K_i$ values for boronic “fast on – fast off” BLIs.

Determination of $k_2/K$ inactivation constant for various enzymes. Inactivation kinetic parameters were determined by the reporter substrate method (32) for slow-tight binding inhibitor kinetic scheme.

$$K = k_2$$

$$E + I \leftrightarrow EI \leftrightarrow EI^*$$

$k_2$

Enzyme was quickly mixed with 100 $\mu$M NCF and various concentrations of inhibitors in reaction buffer and absorbance at 490 nm was measured immediately every two seconds for 600 seconds on SpectraMax plate reader (“Molecular Devices”) at 490 nm. Resulting progression curves of OD$_{490}$ vs time at various BLI concentrations were imported into Prism software (“GraphPad”) and pseudo first-order rate constants $k_{obs}$ were calculated using the following equation

$$P = V_s*(1-e^{-k_{obs}*t})/k_{obs},$$

where $V_s$ - uninhibited enzyme rate. $k_{obs}$ values calculated at various BLI concentrations were fitted in the following equation

$$k_{obs} = k_2 + k_2/K*[I]/(1+[NCF]/K_m,NCF),$$

where $k_2/K$ - inactivation constant

$[I]$ – inhibitor concentration

$[NCF]$ – nitrocefin concentration

$K_m,NCF$ - Michaelis constant of NCF for given enzyme
Stoichiometry of beta-lactamases inhibition by BLIs. Purified enzyme at 1 µM concentration in buffer A was mixed with BLI at BLI:enzyme ratio varying from 128 to 0.0625. After 30 minutes incubation at room temperature, reaction mixture was diluted 100-fold and enzyme activity was measured with nitrocefin as described above. Stoichiometry of inhibition was determined as a minimal BLI:enzyme ratio reducing enzyme activity by at least 90%.

Determination of $k_{off}$ rates of enzyme activity recovery after inhibition by BLIs. Enzyme at 1 µM concentration in buffer A was mixed with BLI at 8-fold higher concentration than its stoichiometry ratio (determined in preliminary stoichiometry experiments). After 30 minutes incubation at 37°C, reaction mixture was diluted from 1000-fold to 10000-fold (depending on enzyme) in buffer A and 100 µl of diluted enzyme was mixed with 100 µl of 400 µM nitrocefin in reaction buffer. Absorbance at 490 nm was recorded for four hours each minute at 37°C. Resulting reaction profiles were fitted into the following equation using “Prizm” software (GraphPad) to obtain $k_{off}$ values:

$$P = V_s * t + (V_o - V_s) * (1 - e^{-k_{off} * t}) / k_{off},$$

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 nitrocefin only

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

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Tables

**Table 1. IC_{50} values (in nM) of β-lactamases inhibition by QPX7728 and comparator BLIs**

| Enzyme | Class | CARB | Vaborbactam | Avibactam | Relebactam | QPX7728 |
|--------|-------|------|-------------|-----------|------------|---------|
| KPC-2  | A     | yes  | 110 ± 30    | 22 ± 6    | 82 ± 17    | 2.9 ± 0.4 |
| CTX-M-14 | A   | no   | 110 ± 40    | 1.4 ± 0.4 | 34 ± 10    | 0.94 ± 0.2 |
| CTX-M-15 | A   | no   | 92 ± 13     | 0.56 ± 0.25 | ND         | 1.2 ± 0.1 |
| SHV-12 | A     | no   | 56 ± 11     | 0.61 ± 0.19 | ND         | 1.9 ± 0.6 |
| TEM-10 | A     | no   | 470 ± 150    | 4.3 ± 1.2 | 160 ± 20    | 2.2 ± 0.8 |
| TEM-43 | A     | no   | 2.2 ± 0.4 x 10^3 | ND     | ND         | 3.0 ± 0.1 |
| P99    | C     | no   | 88 ± 38     | 26 ± 7    | 36 ± 4     | 22 ± 6   |
| OXA-48 | D     | yes  | 6.9 ± 2.3 x 10^3 | 180 ± 50   | 9 ± 0.3 x 10^5 | 1.1 ± 0.4 |
| OXA-23 | D     | yes  | 1.2 ± 0.2 x 10^4 | 3.1 ± 0.6 x 10^3 | ND     | 1.2 ± 0.4 |
| NDM-1* | B     | yes  | >1.6 x 10^5  | >1.6 x 10^5 | >1.6 x 10^5 | 55 ± 25   |
| VIM-1  | B     | yes  | >1.6 x 10^5  | >1.6 x 10^5 | >1.6 x 10^5 | 14 ± 4    |
| IMP-1* | B     | yes  | >1.6 x 10^5  | >1.6 x 10^5 | >1.6 x 10^5 | 610 ± 70   |
| Enzyme   | $K_{d}$ (nM) | $k_2$ (M$^{-1}$ s$^{-1}$) | $k_{off}$ (s$^{-1}$) | Residence time, min | Stoichiometry |
|----------|-------------|--------------------------|---------------------|---------------------|--------------|
| KPC-2    | 3.6 ± 0.1 × 10$^5$ | 9.0 ± 1.4 × 10$^5$ | 189 ± 31 | 0.25 ± 0.03 | 1             |
| KPC-3    | 4.1 ± 1.0 × 10$^5$ | 1.26 ± 0.07 × 10$^4$ | 133 ± 8 | 0.31 ± 0.06 | 1             |
| BKC-1    | 1.82 ± 0.04 × 10$^6$ | 1.1 ± 0.1 × 10$^4$ | 154 ± 14 | 0.060 ± 0.005 | 1             |
| FRI-1    | 1.15 ± 0.03 × 10$^6$ | 1.2 ± 0.2 × 10$^4$ | 138 ± 24 | 0.11 ± 0.01 | 1             |
| SME-2    | 1.2 ± 0.1 × 10$^6$ | 1.8 ± 0.2 × 10$^4$ | 94 ± 11 | 0.15 ± 0.02 | 1             |
| CTX-M-15 | 6.9 ± 0.6 × 10$^6$ | 8.0 ± 1.0 × 10$^5$ | 220 ± 33 | 0.11 ± 0.02 | 1             |
| SHV-12   | 1.1 ± 0.2 × 10$^6$ | 3.0 ± 0.2 × 10$^4$ | 5.5 ± 0.3 | 28 ± 4 | 1             |
| TEM-43   | 1.9 ± 0.3 × 10$^6$ | 3.2 ± 0.2 × 10$^4$ | 53 ± 3 | 0.17 ± 0.02 | 1             |
| P99      | 6.3 ± 0.7 × 10$^4$ | 3.3 ± 0.3 × 10$^4$ | 506 ± 51 | 0.53 ± 0.06 | 1             |
| OXA-48   | 2.75 ± 0.09 × 10$^6$ | 3.6 ± 0.2 × 10$^4$ | 47 ± 3 | 0.13 ± 0.01 | 1             |
| OXA-23   | 9.9 ± 0.6 × 10$^6$ | 1.6 ± 0.2 × 10$^5$ | 11 ± 2 | 1.6 ± 0.2 | 2             |
| OXA-24   | 1.5 ± 0.2 × 10$^6$ | 9.0 ± 1.0 × 10$^4$ | 20 ± 3 | 0.58 ± 0.10 | 1             |
| OXA-58   | 1.07 ± 0.08 × 10$^6$ | 3.5 ± 0.3 × 10$^4$ | 4.8 ± 0.5 | 3.2 ± 0.3 | 1             |
| NDM-1    | ND           | ND           | ND           | 32 ± 14 | ND           |
| VIM-1    | ND           | ND           | ND           | 7.5 ± 2.1 | ND           |
| IMP-1    | ND           | ND           | ND           | 240 ± 30 | ND           |

*K_{d} values for serine beta-lactamases were derived from the ratio of $k_{off}$ and $k_2/K$. *K_{d} values for MBLs were based on K_{i}.*

B: yes

* - Imipenem was used as a substrate.

CARB, carbapenemase activity; ND, not done.
Figure 1. Beta-lactamase Inhibitors

Figure 2. Kinetic profiles of inactivation of serine and metallo beta-lactamases by QPX7728.

A, serine beta-lactamases; B, metallo beta-lactamases QPX7728 at indicated concentration (in µM) was quickly mixed with each enzyme and 100 µM nitrocefin and absorbance at 490 nm was recorded immediately every 2 seconds using plate reader.
Enzymes at 1 µM concentration were mixed with QPX7728 at 8 µM concentration and incubated for 30 minutes. After appropriate dilution, 200 µM nitrocefin was added to the reaction mixture and absorbance at 490 nm was recorded every 10 seconds using plate reader. Reaction without addition of BLI was also recorded and used to calculate uninhibited enzyme velocity $V_s$. 

Figure 3. Kinetic profiles of activity recovery of various beta-lactamases after inhibition by QPX7728 using jump dilution method.
Figure 4. Lineweaver-Burk plots of VIM-1 (left panel) and NDM-1 (right panel) inhibition by QPX7728.

Enzymes were mixed with various concentrations of nitrocefin substrate (x axis) and indicated amount of QPX7728 (in brackets, µM) and reaction profiles were recorded for 10 minutes at 490 nm. Initial rates of reaction were calculated, and corresponding reciprocal values were plotted against 1/S.

Figure 5. Superposition of QPX7728 and the typical core ring system of beta-lactam antibiotics

QPX7728 in light blue carbon atoms and the typical core ring system of beta-lactam in crème carbon atoms. Close correspondence of molecular volumes and other features between the inhibitor and the substrate core can be observed. Molecular alignment and visualization performed in ICM-Pro (Molsoft, San Diego).