The Impact of Intrinsic Resistance Mechanisms on Potency of QPX7728, a New Ultra-Broad-Spectrum Beta-lactamase Inhibitor of Serine and Metallo Beta-Lactamases in Enterobacteriaceae, Pseudomonas aeruginosa, and Acinetobacter baumannii.

Short title: Intrinsic Resistance Mechanisms and QPX7728

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ABSTRACT

QPX7728 is an ultra-broad-spectrum boronic acid beta-lactamase inhibitor that demonstrates inhibition of key serine and metallo beta-lactamases at a nano molar range in biochemical assays with purified enzymes. The broad-spectrum inhibitory activity of QPX7728 observed in biochemical experiments translates into enhancement of the potency of many beta-lactams against strains of target pathogens producing beta-lactamases. The impact of bacterial efflux and permeability on inhibitory potency were determined using isogenic panels of KPC-3 producing isogenic strains of *K. pneumoniae* and *P. aeruginosa* and OXA-23-producing strains of *A. baumannii* with various combinations of efflux and porin mutations. QPX7728 was minimally affected by multi-drug resistance efflux pumps in either *Enterobacteriaceae*, or in non-fermenters such as *P. aeruginosa* or *A. baumannii*. In *P. aeruginosa*, the potency of QPX7728 was further enhanced when the outer membrane is permeabilized. The potency of QPX7728 in *P. aeruginosa* is not affected by inactivation of the carbapenem porin OprD. While changes in OmpK36 (but not OmpK35) reduced the potency of QPX7728 (8-16-fold), QPX7728 (4 µg/ml) nevertheless completely reversed KPC-mediated meropenem resistance in strains with porin mutations, consistent with a lesser effect of these mutations on the potency of QPX7728 compared to other mutations. The ultra-broad-spectrum beta-lactamase inhibition profile combined with enhancement of the activity of multiple beta-lactam antibiotics with varying sensitivity to the intrinsic resistance mechanisms of efflux and permeability indicate QPX7728 is a useful inhibitor for use with multiple beta-lactam antibiotics.

Key words: QPX7728, serine beta-lactamases, metallo-beta-lactamases, efflux, porins

INTRODUCTION
QPX7728 (Figure 1) is a new cyclic boronic acid beta-lactamase inhibitor (BLI) (1) with inhibition of key serine and metallo-beta-lactamases at a nanomolar range in biochemical assays with purified enzymes (2). QPX7728 inhibits class A ESBLs and carbapenemases such as KPC as well as class C P99 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 as well as MBLs such as NDM-1, VIM-1 and IMP-1.

QPX7728 differs from investigational beta-lactamase inhibitors in clinical development such as the avibactam analog durlobactam and the bicyclic boronate taniborbactam (3). Durlobactam spectrum includes OXA enzymes from Acinetobacter and its potency to inhibit these enzymes appears to be similar to that of QPX7728 (based on Kd values) but it does not inhibit Class B metallo beta-lactamases (11, 12). Taniborbactam and QPX7728 inhibit NDM and VIM metallo beta-lactamases with a similar potency, but taniborbactam lacks inhibitory activity of OXA carbapenemases from Acinetobacter (4, 5).

The broad-spectrum inhibitory activity of QPX7728 observed in biochemical experiments translates into enhancement of the activity of many beta-lactams against extended beta-lactamase and carbapenemase (serine and metallo) producing strains of Enterobacteriaceae, carbapenemase (OXA) producing strains of Acinetobacter baumannii and multi-drug resistant strains of Pseudomonas aeruginosa (1). This inhibitory activity of QPX7728 can also be demonstrated in mouse thigh and lung infection models of infections where meropenem showed efficacy against KPC-producing strains of Enterobacteriaceae (K. pneumoniae and E. cloacae), OXA-23-producing strains of A. baumannii and multidrug resistant strains of P. aeruginosa that did not respond to meropenem alone (6).

Gram-negative bacteria possess multiple intrinsic mechanisms that modulate activity of various antibiotics, including beta-lactams (7, 8). Reduced uptake across the outer membrane, increased efflux by multidrug resistance pumps and a combination of these mechanisms results in decreased susceptibility to beta-lactam antibiotics that cannot be reversed with beta-lactamase inhibitors. In Enterobacteriaceae this occurs due to mutations in major porins OmpF/OmpK35 and OmpC/OmpK36 (9-12) and increased efflux
out of cells by multidrug resistant pumps such as AcrAB (13). *P. aeruginosa* in particular has multiple intrinsic resistance mechanisms that impact multiple antibiotics (14), notably with several multi-drug resistant pumps (15) with MexAB-OprM having varying effects on beta-lactam antibiotics. Mutations in the porin OprD (16) are specifically associated with reduced susceptibility to carbapenems. AdeABC and AdeIJK MDR efflux pumps from *A. baumannii* are implicated in general defense (17). Similar to beta-lactams, the potency of beta-lactamase inhibitors also can be also affected by the same general resistance mechanisms (18-21).

The objective of this study was to investigate the impact of general intrinsic resistance mechanisms of gram-negative bacteria on the inhibitory potency of QPX7728. The impact of porin and efflux mutations was investigated in several target bacteria using various microbiological assays. The choice of an antibiotic in each assay presented here is driven the intrinsic resistance mechanism being probed for QPX7728 where the partner antibiotic tested may be affected to little or no extent. Studies of QPX7728 in these defined systems should help to better understand its behavior against clinical isolates with its complicated combinations of various intrinsic resistance mechanisms.

RESULTS AND DISCUSSION

The outer membrane porin OmpK36, and to a lesser degree efflux, modulate the whole-cell BLI activity of QPX7728 in *K. pneumoniae*
A set of isogenic KPC-producing strains of *K. pneumoniae* with various combinations of efflux and porin mutations was used to investigate the contribution of porins and efflux to the BLI activity of QPX7728 in *K. pneumoniae* (22). Meropenem was used as a tool antibiotic. Varying concentrations of QPX7728 or vaborbactam used to assess the effect on meropenem MICs in checkerboard experiments. As the parameter for comparison of BLI potency, we used the concentration of a BLI required to reduce the meropenem MIC to the level seen in the parent strain that lacks KPC. This value, $PV_{\text{max}}$, (maximal potentiation value) corresponds to the complete inhibition of the enzyme.

Inactivation of *ompK35* alone did not increase the QPX7728 $PV_{\text{max}}$ values (compare KPM2601 vs KPM1271), while inactivation of *ompK36* alone increased the QPX7728 $PV_{\text{max}}$ value 8-16-fold, from 0.125 µg/ml to 1-2 µg/ml (compare KPM2599 and KPM2067 vs KPM1271) (Table 1; see Supplementary Table S2 for the full concentration-response of meropenem MIC to varying concentrations of QPX7728 and vaborbactam). Vaborbactam $PV_{\text{max}}$ values were increased 4-fold and 64-fold by inactivation of OmpK35 (to 1 µg/ml) and OmpK36 alone (to 16 µg/ml), respectively. Note, that both BLIs have similar potency against the wild type strain PAM1271. Inactivation of *ompK35* in the strain that already lacked OmpK36 (KPM2631 vs KPM2067) did not increase the $PV_{\text{max}}$ value for QPX7728 (it remained at 2 µg/ml), while the vaborbactam $PV_{\text{max}}$ value was increased 4-fold, to 64 µg/ml. Inactivation of both Ompk35 and OmpK36 reduced vaborbactam potency 256-fold; only a 16-fold effect (driven by OmpK36 alone) was observed for QPX7728.
Strains of *K. pneumoniae* that carry a variant of OmpK36 with a duplication of two amino acids, Gly134Asp135 in the L3 loop (GD repeat) results in a functionally constricted inner channel; these variants are being increasingly reported in clinical settings (23). PV\textsubscript{max} value of QPX7728 for meropenem potentiation for the clinical isolate KP1074 (2 µg/ml, OmpK36 with “GD repeat”) was 8-fold higher compared to that for KP1004 (0.25 µg/ml, wild-type OmpK36). In the case of vaborbactam, PV\textsubscript{max} values were increased 16-fold, from 2 µg/ml to 32 µg/ml. While these strains are not truly isogenic, the “GD repeat” in KP1074 is the most likely reason for this difference. Based on PV\textsubscript{max}, the narrowing of the OmpK36 aperture had the same effect on potency of QPX7728 as the complete inactivation of OmpK36.

Strains KPM1272 and KPM1271 were used to assess the effect of the ramR mutation on the meropenem potentiation activity of QPX7728. Inactivation of ramR (KPM1272) results in both ~3-4-fold acrAB overexpression and ~10-fold ompK35 downregulation (22). The QPX7728 PV\textsubscript{max} value was 2-fold higher in KPM1272 than in KPM1271; the PV\textsubscript{max} value for KPM1272 was 2-fold higher relative to that of KPM2601, the mutant that lacks OmpK35. In addition, a 2-fold effect of AcrAB overexpression on QPX7728 inhibitory potency was seen in the strains that lacked (or had low expression) of both OmpK35 and OmpK36 (KPM2631 vs KPM2818 and KPM2631 vs KPM2965). These data indicate that the increased efflux due to the AcrAB efflux pump has a small effect on QPX7728 potentiation of meropenem in *K. pneumoniae*.

In summary, complete or partial inactivation of the major porin OmpK36 results in reduced whole-cell BLI potency with QPX7728. OmpK36 deficiency appears to affect QPX7728 to a significantly lesser degree than vaborbactam (16-fold vs 64-fold reduction in PV\textsubscript{max} for QPX7728 and vaborbactam, respectively). In contrast to vaborbactam, the BLI potency of QPX7728 is not affected by mutations in OmpK35. Consequently, there is no additional loss in the inhibitory potency of QPX7728 in strains with double porin mutations, whereas vaborbactam potency was further reduced (256-fold decrease).

Increased efflux conferred ca. two-fold decrease in potency (increase in PV\textsubscript{max}) of QPX7728, so that the highest concentration of QPX7728 associated with a complete inhibition of KPC activity in whole cells was observed in the strains with a combination of increased efflux and defects in OmpK36. Even though a
The inhibitory activity of QPX7728 is affected by the outer membrane barrier in *P. aeruginosa*

The contribution of the outer membrane barrier of *P. aeruginosa* to the BLI activity of QPX7728 was studied using strains with varying efflux activity and the outer membrane permeabilizing agent...
PMBN. QPX7728 $PV_{\text{max}}$ for meropenem potentiation was determined in the KPC-2 producing strain PAM4135 that lacks the major constitutively expressed efflux pump MexAB-OprM. MICs were determined in the presence of increasing concentrations of the outer membrane permeabilizing agent PMBN (27) (Table 2). PMBN at 2.5 µg/ml and 5 µg/ml reduced the MIC of meropenem two-fold and 8-fold, respectively. In the presence of increasing concentrations of PMBN, QPX7728 $PV_{\text{max}}$ values were reduced 4-fold and 16-fold. These data show that like many other small molecule antibiotics, the outer membrane of *P. aeruginosa* does present a barrier to entry of QPX7728. Low permeability of the outer membrane of *P. aeruginosa* appears to be responsible for the lower BLI potency of QPX7728 in *P. aeruginosa* as compared to that in *K. pneumoniae*: QPX7728 $PV_{\text{max}}$ value for KPC inhibition 2-8 µg/ml in *P. aeruginosa* vs 0.125-0.25 µg/ml in *K. pneumoniae*. In the presence of 5 µg/ml PMBN, QPX7728 $PV_{\text{max}}$ value of KPC inhibition in *P. aeruginosa* is the same as $PV_{\text{max}}$ of KPC inhibition in *K. pneumoniae*, (0.125 µg/ml). Similarly, the QPX7728 $PV_{\text{max}}$ values for KPC inhibition in *P. aeruginosa*, are also similar to those in *K. pneumoniae* that lack functional porins. The $PV_{\text{max}}$ values will be taken into consideration when translating the BLI potency observed in microbiological experiments into exposures that are expected to be associated with BLI activity in vivo against target pathogens. It also means that the outer membrane permeabilization might be considered as a future strategy to increase the whole cell antibiotic potentiation activity of QPX7728.

**The BLI activity of QPX7728 is minimally affected by MDR efflux in *P. aeruginosa* and *A. baumannii***

A set of isogenic KPC-producing strains of *P. aeruginosa* overexpressing or lacking major efflux pumps (28, 29) was used to assess the effect of efflux on the inhibitory activity of QPX7728 in *P. aeruginosa*. Biapenem was chosen as the reporter antibiotic because its activity is minimally affected by efflux (30). Vaborbactam was used as a comparator BLI. Biapenem MICs were determined in the presence of increasing concentrations of BLIs. BLI potency was expressed as either $PV_{\text{max}}$ (see above) or $PV_{50}$ (31); $PV_{50}$ is defined as the concentration of a BLI achieving 50% of the antibiotic potentiation
effect, which is determined by the midpoint of the difference between the partner antibiotic MIC for the beta-lactase producing strain compared to the MIC of the vector only strain (MIC middle point calculated as a geometric mean of the above MICs) (Table 3). Overexpression of the MexAB-OprM efflux pump increased QPX7728 PV_{50} and PV_{max} values two-fold, from 1 µg/ml to 2 µg/ml and from 4 µg/ml to 8 µg/ml (compare PAM4126 vs PAM4224). No change in QPX7728 potency was observed when this pump was inactivated due to mutations in mexA (PAM4365) and oprM (PAM4135). In contrast, vaborbactam potency was strongly affected by either increased or decreased MexAB-OprM-mediated efflux: overexpression of MexAB-OprM reduced vaborbactam potency 8-fold (PV_{50} increased from 8 µg/ml to 64 µg/ml), while MexAB-OprM inactivation increased its potency 16-fold (PV_{50} decreased from 8 µg/ml to 0.5 µg/ml). QPX7728 potency was not affected by overexpression of the MexEF-OprN efflux pump, whereas vaborbactam potency was significantly reduced (>8-fold increase in the PV_{50} value to >64 µg/ml; comparison of PAM4132 vs PAM4224). Neither MexXY-OprM nor MexCD-OprJ had a significant effect on the activity of either BLI.

These experiments demonstrated that the BLI activity of QPX7728 is minimally affected by the activity of major MDR efflux pumps from *P. aeruginosa*, representing a significant improvement over the earlier generation boronate BLI vaborbactam. As this result was obtained using KPC as a reporter beta-lactamase, we wanted to test the validity of this conclusion using an additional beta-lactamase and more antibiotics. Overexpression of chromosomal AmpC is one of the more prevalent mechanisms of beta-lactam resistance in *P. aeruginosa* (14). Hence, we evaluated the MICs of several beta-lactam antibiotics in combination with QPX7728 against isogenic strains of *P. aeruginosa* overexpressing the chromosomal AmpC beta-lactamase (PDC-1) with or without concomitant overexpression of the major efflux pump, MexAB-OprM. QPX7728 was tested at 4 µg/ml and 8 µg/ml which corresponds to the PV_{max} of QPX7728 for the inhibition of KPC in the strain overexpressing MexAB-OprM.

The lowest MIC increase due to AmpC overexpression, 4-fold (from 0.5 µg/ml to 2 µg/ml), was observed for meropenem, and the highest (>64-fold; from 4 µg/ml to >256 µg/ml) for piperacillin (PAM1020 vs PAM2156) (Table 4). Ceftolozane MICs were not affected by MexAB-OprM-mediated
efflux, and other antibiotics were affected 4-8-fold (PAM1020 vs PAM1032). Overexpression of AmpC in the strain with MexAB-OprM overexpression did not significantly increase the MIC values of meropenem; MIC values for other antibiotics were increased 4 to >16-fold (PAM1032 vs PAM2005).

QPX7728 (tested at either 4 µg/ml or 8 µg/ml) had no effect on antibiotic MICs against PAM1020 and PAM1032; however, when tested against the strain PAM2156 that overexpressed AmpC but had a basal level of MexAB-OprM, QPX7728 at both concentrations reduced the MIC values of all tested antibiotics except piperacillin to the level observed for PAM1020, indicating complete inhibition of AmpC.

Piperacillin MIC tested with QPX7728 at 4 µg/ml was 4-fold higher compared to that of PAM1020 (16 µg/ml vs 4 µg/ml); increasing QPX7728 concentration to 8 µg/ml resulted in a complete reversion of resistance, presumably due to complete inhibition of AmpC. For the strain PAM2005 overexpressing both AmpC and MexAB-OprM MICs for all antibiotics (except piperacillin) with QPX7728 at both concentrations were either the same or not more than two-fold higher compared to that of PAM1032, overexpressing MexAB-OprM alone. Piperacillin MIC was 8-fold and 2-fold higher than that of PAM1032 with QPX7728 at 4 µg/ml and 8 µg/ml, respectively, indicating a very similar dose response for potentiation compared to that observed for PAM2156 with a basal level of MexAB-OprM. These experiments confirmed that the inhibitory potency of QPX7728 to reverse resistance to multiple anti-pseudomonal beta-lactams was minimally affected by overexpression of the major efflux pumps in *P. aeruginosa*. This important feature differentiates QPX7728 from avibactam (32) and vaborbactam, which are substrates of efflux pumps and demonstrate reduced potency in strains with increased efflux.

Combining QPX7728 with a beta-lactam antibiotic not affected by efflux will ensure that the potency of a combination agent not be affected by efflux as well.

QPX7728 is a potent inhibitor of Class D carbapenemases from *A. baumannii*. Non-beta-lactamase-mediated resistance mechanisms in Acinetobacter were assessed to determine the potency of various beta-lactam/QPX7728 combinations. The impact of MDR efflux on antibiotic potentiation activity of QPX7728 in *A. baumannii* (17) was evaluated using a panel of isogenic OXA-23-producing strains with wild-type (ACM1565) or increased expression of two major efflux operons, adeIJK.
(ACM1566) and adeABC (ACM1567). QPX7728 PV$_{50}$ and PV$_{\text{max}}$ were determined using biapenem (not a substrate of efflux pumps) or meropenem potentiation experiments. QPX7728 PV$_{\text{max}}$ values for OXA-23 inhibition in A. baumannii were in the range of 2-4 µg/ml. When meropenem, a substrate of AdeIJK was used as a reporter antibiotic, overexpression of AdeIJK increased meropenem MIC with QPX7728 at various concentrations ca. 2-32-fold. No changes in QPX7728 PV$_{\text{max}}$ values in either biapenem or meropenem checkerboard experiments was detected in strains overexpressing major efflux operons, indicating that efflux has a minimal effect on the inhibitory activity of QPX7728 in A. baumannii (Table 5).

SUMMARY

QPX7728 is a new boronate BLI with potent inhibitory activity against both serine and metallo beta-lactamases. The broad-spectrum inhibitory activity of QPX7728 previously observed in cell-free biochemical experiments using purified enzymes translates into enhancement of the activity of many beta-lactams against strains of target pathogens producing beta-lactamases (2, other paper!).

The potent inhibitory activity of QPX7728 in whole-cells is driven in part by lack of efflux by major transporters from gram-negative bacteria at concentrations that are relevant for beta-lactamase inhibition. Lack of efflux of QPX7728 is particularly important for inhibitory activity in P. aeruginosa and represents a significant improvement over the earlier boronate BLI vaborbactam. Mutations in outer membrane porin proteins of Enterobacteriaceae are associated with reduced potency of many antibiotics and beta-lactamase inhibitors. The potency of QPX7728 in Enterobacteriaceae is affected much less by the inactivation of the major general porins, OmpK35/OmpF and OmpK36/OmpC as compared to the boronate inhibitor vaborbactam.

The potent, ultra-broad-spectrum BLI activity of QPX7728 shown with multiple beta-lactam antibiotics with varying sensitivity to beta-lactamases as well as intrinsic resistance mechanisms makes it an ideal candidate for multiple product development strategies. Conventional approaches for product
configurations include development of a fixed combination beta-lactam/beta-lactamase inhibitor for which there is a well-established regulatory path. An important limitation of this strategy is identifying a partner beta-lactam that in combination with the BLI has the best overall activity against most but perhaps not all target pathogens with different mixtures of resistance mechanisms. Another approach would be development as a “stand-alone” drug product that would be co-administered with different existing beta-lactam antibiotics depending on the mechanisms present in the specific pathogen. This approach has several clinical and regulatory implications but could be an important step towards individualized treatment of drug-resistant pathogens through taking into account local epidemiology, patient factors, and antibiotic stewardship. The multiple benefits of this strategy should encourage the establishment of a defined path for future regulatory approval.

MATERIAL AND METHODS

Panels of engineered bacterial strains containing various combinations of porin and efflux mutations. The efflux/porin isogenic panels of *K. pneumoniae*, *P. aeruginosa* and *A. baumanii* were constructed to evaluate the impact of various molecular determinants on the whole-cell antibiotic potentiation activity of QPX7728.

The construction of a panel of isogenic KPC-3 producing strains (carried on a naturally occurring plasmid pKpQIL) of *K. pneumoniae* with various combinations of porin (*ompK35 and ompK36*) and efflux (*acrAB-tolC*) mutations was described earlier (22). The panel of isogenic KPC-2-producing strains of *P. aeruginosa* overexpressing or lacking MDR RND efflux pumps, MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, and producing or lacking carbapenem porin OprD, was constructed by transforming the plasmid pUCP24-KPC-2 into various mutants. The panel of isogenic OXA-23-producing strains of *A. baumannii* overexpressing MDR RND efflux pumps, AdeABC and AdeIJK, was constructed by conjugating the natural plasmid that carries OXA-23 from the clinical isolate AB1177 into various efflux mutants.

A detailed description of all strains used in this study is provided in the **Supplementary Table S1**.
Conjugation in *Acinetobacter baumannii*. First, rifampicin-resistant mutants of recipient strains were isolated on LB (Luria-Bertani) agar containing rifampicin at 40 \( \mu \text{g/ml} \). Next, both donor and recipient strains were grown overnight in LB at 37°C with aeration. Next day, the donor and recipient cultures (100\( \mu \text{l} \) each) were mixed and pelleted by centrifugation for 1 minute at room temperature. The cells were resuspended in 40\( \mu \text{l} \) of LB and spotted onto an LB agar plate without antibiotics. Recipient-only and donor-only cultures were similarly spotted on LB plates as negative controls. The plates were incubated at 37°C for 4-5 hours, and cells were collected and resuspended in 0.5 ml of LB medium to OD\(_{600}\) = 0.1-0.5. The 0.05-0.1\( \mu \text{l} \) of cell suspension was plated on a plate containing rifampicin and meropenem at 40 \( \mu \text{g/ml} \) and 2 \( \mu \text{g/ml} \), respectively. Transconjugants were verified by PCR and DNA sequencing.

Transduction in *P. aeruginosa* using phage F116. Lysates of the donor strains were prepared using the soft agar method with modifications (33). Briefly, 0.1 ml of a culture grown overnight in LB broth was mixed with 1 ml of the F116 phage suspension with a titer of \( 5 \times 10^5 \) plaque-forming units per ml in LB broth. The mixtures were incubated at 37°C for 15 minutes, and 2 ml of the melt 0.9% LB agar kept at 45°C was added to each mixture, mixed and immediately poured onto a freshly prepared regular LB agar plate. The plates were incubated right-side up overnight at 30°C. The top soft agar layer was scrapped after adding 5-ml of LB broth and poured into a centrifuge tube. Two drops of chloroform were added to each tube. The tubes were vortexed vigorously for 1 minute, kept at 4°C for 1 hour and centrifuged at 5,000 rpm for 5 minutes at 4°C. The supernatant was sterilized by filtration. For transduction, recipient cells were grown in LB to OD\(_{600}\) 1, and 0.3 ml of the culture was mixed with 15 \( \mu \text{l} \) of a phage lysate prepared from a donor strain. After incubation at 37°C for 20 minutes, the cells were pelleted by centrifugation for 1 minute and washed once with 1 ml of TNM buffer (0.01 M Tris-HCl, pH7.4, 0.15 M NaCl, 0.01 M MgSO\(_4\)). The cells were resuspended in 0.1 ml of TNM buffer and spread onto an LB agar plate. After 2 h of incubation at 37°C, 0.4 ml of LB broth was added to the plate and the cells were
collected and directly transferred to an LB agar plate containing the selecting antibiotic. Transductants were verified by PCR and DNA sequencing.

**ampC gene expression.** A single colony from an overnight plate was inoculated into cation-adjusted Mueller-Hinton broth (CA-MHB) and grown at 37°C with aeration to OD600 of ~0.7. 1.5 ml of cell culture was pelleted by centrifugation and total RNA was isolated using an Ambion Ribopure-Bacteria RNA Isolation kit (ThermoFisher, San Diego, CA). Residual DNA in the RNA samples was removed by treatment with DNase I, according to the manufacturer’s instructions. Reverse transcription (RT) was performed using TaqMan® Reverse Transcriptase Reagents kit (ThermoFisher, San Diego, CA) and a mixture of reverse primers for the ampC (PA-ampC-R 5’ TGAAGGTCTTGCTCACCGAG 3’), and the polA (PA-polA-R 5’ATCTGGTCGAAGGTCAGTTG 3’) genes, each at a final concentration 0.5 µM.

The RT reaction mixture was diluted 10-fold and used as a template in quantitative PCR (qPCR) on an ABIPrism 7000 Sequence Detection System (Applied Biosystems) using SYBR® Select Master Mix (ThermoFisher). The total volume of each reaction was 20 µl, including 9 µl of diluted RT reaction mixture, 10 µl of SYBR® Select Master Mix (2x) and 1 µl of a qPCR primer pair mix (PA-ampC-F 5’ GAAAGGAGAACCGCATTAC 3’ and PA-ampC-R 5’ TGAAGGTCTTGCTCACCGAG 3’ ) and PA-polA-F 5’ ATCCGAAGAAGCTCAAGGTC 3’ and PA-polA-R 5’ATCTGGTCGAAGGTCAGTTG 3’

at a final concentration of 0.5 µM. The qPCR was run in duplicate under the following thermal cycling conditions: 50°C for 2 min, 95°C for 5 min, and followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec, and 70°C for 45 sec. The housekeeping gene polA, encoding DNA polymerase I, was used as internal control. The CT value of ampC gene was normalized with that of polA gene of the same strain. To calculate the ampC gene transcription level in a test strain relative to that in the wild type strain PAM1020, the normalized CT value of PAM1020 was subtracted from that of the test strain, and the difference (ΔCT) was used as a logarithmic power (base=2) to calculate the relative level of mRNA.
**Antimicrobial susceptibility testing.** Bacterial isolates were subjected to broth microdilution susceptibility testing, performed according to Clinical and Laboratory Standards Institute (CLSI) methods (34), using panels prepared in-house. A checkerboard assay conforming to the Moody procedures in Clinical Microbiology Procedures Handbook (35) was used to evaluate the effect of varying concentrations of QPX7728 or vaborbactam on MIC to various antibiotics. \( PV_{50} \) and \( PV_{\text{max}} \) (PV stands for the potentiation value) values were used to define the potency of beta-lactamase inhibitors (31). \( PV_{50} \) was defined as a minimal concentration of a BLI to achieve 50% of antibiotic potentiation effect or a concentration of a BLI to reduce antibiotic MIC to the middle point of the MIC range between the MIC of the beta-lactamase producing strain and the corresponding beta-lactamase lacking strain. The MIC middle point is a geometric mean of the antibiotic MIC values for the beta-lactamase-producing and the beta-lactamase lacking strain and is calculated as the square root of the product of the antibiotic MIC values for the beta-lactamase-producing and the beta-lactamase lacking strain. 

\[ PV_{\text{max}} \] was defined as minimal potentiating concentration of the BLI to reduce antibiotic MIC to the level seen in the parent strain that lacks beta-lactamase (KPC) (corresponding to complete inhibition of KPC).

Meropenem was purchased from Sandoz, all other antibiotics were from Sigma Aldrich. QPC7728 and vaborbactam were synthesized at Qpex Biopharma, San Diego, CA.

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Table 1: The potency of QPX7728 (PV\textsubscript{max}) to enhance the activity of meropenem against isogenic strains of KPC-3-producing *K. pneumoniae* with various combinations of efflux and porin mutations

| KPC-3 producing strain\(^1\) | Recipient Strain | Genotype/Construction | OmpK35 | OmpK36 | AcrAB | Meropenem MIC (µg/ml) | QPX PV\textsubscript{max}\(^2\) (µg/ml) | VAB PV\textsubscript{max}\(^2\) (µg/ml) |
|-----------------------------|------------------|------------------------|--------|--------|--------|----------------------|--------------------------|--------------------------|
| KPM1271                    | KPM1026a         | Wild type              | FL     | FL     | BL     | 16 0.016 0.06 0.06   | 0.125 0.25                |             |
| KPM2801                    | KPM2800          | ΔompK35                | NF     | FL     | BL     | 32 0.016 0.06 0.06   | 0.125 1                   |             |
| KPM2599                    | KPM2592          | ΔompK36                | FL     | NF     | BL     | 32 0.03 0.25 0.06   | 1 16                      |             |
| KPM2040                    | KPM2040          | ompK36\_fs             | FL     | NF     | BL     | 64 0.03 0.5 0.125  | 2 16                      |             |
| KPM2613                    | KPM2613          | ΔompK35 ΔompK36        | NF     | NF     | BL     | 256 0.25 4 0.25    | 2 64                      |             |
| KPM2966                    | KPM2966          | ompK36\_fs             | NF     | NF     | Up     | 256 0.5 8 0.5     | 4 64                      |             |
| KPM1272                    | KPM1027          | ramR\_fs               | Down   | FL     | Up     | 16 0.016 0.06 0.06 | 0.25 2                    |             |
| KPM2818                    | KPM2818          | ramR\_fs               | Down   | NF     | Up     | 256 0.5 8 0.5     | 4 64                      |             |
| KPM1007                    | KPM1007          | ΔacrB                  | FL     | FL     | NF     | ND   ND 0.06       | ND ND                    |             |
| KPM1206                    | KPM1206          | ompK36\_fs             | NF     | FL     | BL     | 32 0.016 0.03 0.03 | 0.25 2                    |             |
| KPM1211                    | KPM1211          | ompK36\_fs ΔompK36\_GD | NF     | GD\(^4\) | BL | 128 0.06 1 0.125 | 2 32                      |             |

\(^1\) All strains produce KPC-3 and TEM-1 encoded in plasmid pKpQIL. Both KPM1026a derivatives and clinical isolates also produce chromosomal SHV enzyme, encoded by *bla*\textsubscript{SHV}-24 and *bla*\textsubscript{SHV}-11, respectively.

\(^2\) PV\textsubscript{max}, minimal potentiating concentration of the BLI to reduce meropenem MIC to the level seen in the parent strain that lacks KPC, which corresponds to complete inhibition of KPC.

\(^3\) QPX, QPX7728 and VAB, vaborbactam at 4 µg/ml

\(^4\) Duplication of two amino acids, Gly134 and Asp135, located within the L3 internal loop and associated with the reduced susceptibility to carbapenems due to constriction of the channel

ompK35\_fs and ompK36\_fs, truncated porins due to the frame-shift mutation; ramR\_fs, truncated ramR due to the stop codon; FL, full length; NF, non-functional; BL, basal level; Up, overexpressed; down, downregulated.
Table 2. The effect of inactivation of the carbapenem-specific porin OprD or the permeabilization of the outer membrane on potency of QPX7728 in P. aeruginosa

| Strain | Genotype | Antibiotic | Antibiotic MIC (µg/ml) in the presence of varied concentrations of QPX7728 (µg/ml) | MIC of vector only (µg/ml) | PV<sub>max</sub> (µg/ml) |
|--------|----------|------------|---------------------------------------------------------------------------------|--------------------------|--------------------------|
| PAM4135 | oprM::Hg  | Cefepime   | 256 64 64 64 32 2 1 0.125 0.125 0.03 0.5 2 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 | 0.03 0.125 0.03 0.125 0.03 0.125 | 0.03 0.125 | 2 |
| PAM4135 | oprM::Hg oprD<sup>2</sup> | Cefepime   | 256 64 64 64 32 2 1 0.125 0.125 0.03 0.5 2 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 | 0.03 0.125 0.03 0.125 0.03 0.125 | 0.03 0.125 | 2 |

The impact of OprD inactivation on BLI potency of QPX7728 was evaluated based on the ability of QPX7728 to reduce cefepime or meropenem MIC in isogenic KPC-producing strains that produced or lacked OprD. The effect of the outer membrane barrier on BLI potency of QPX7728 was evaluated based on the ability of the outer membrane permeabilizing agent, PMBN, to increase BLI potency of QPX7728 in meropenem potentiation assay using the KPC-2 producing strain of P. aeruginosa PAM4135 that lacks MexAB-OprM efflux pump. The potency of QPX7728 was expressed as PV<sub>max</sub>, minimal potentiating concentration of the BLI to reduce antibiotic MIC to the level seen in the parent strain that lacks KPC (corresponding to complete inhibition of KPC).

<sup>2</sup> oprD mutant PAM4756 (oprD::Tn5,aadA126) was selected from PAM4135 on 128 µg/ml of biapenem (4XMIC)

<sup>3</sup> Meropenem/QPX7728 checkerboard plates contained PMBN at a fixed concentration of 2.5 µg/ml and 5 µg/ml

Cefepime and meropenem MIC in the presence of BLIs at PV<sub>max</sub> are marked with green color.

Table 3: The potency of QPX7728 (PV<sub>40</sub> and PV<sub>max</sub>) to enhance the activity of biapenem against isogenic strains of KPC-2-producing P. aeruginosa overexpressing or lacking multidrug resistance RND efflux pumps

| Strain | Genotype | Description | BLI | Biapenem MIC (µg/ml) in the presence of varied concentrations of BLIs (µg/ml) | MIC of the vector only strain (µg/ml) | GM-MIC<sup>2</sup> (µg/ml) | PV<sub>40</sub> (µg/ml) | PV<sub>max</sub> (µg/ml) |
|--------|----------|-------------|-----|---------------------------------------------------------------------------------|-------------------------------------|-----------------|-----------------|-----------------|
| PAM4324 | Wildtype  | QPX7728     | 64  64 32 32 16 2 1 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 | 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 | 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 | 1 1 1 1 1 1 1 1 | PV<sub>max</sub> |
| PAM4135 | oprM::Hg  | QPX7728     | 64  64 32 32 16 2 5 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 | 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 | 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 | 1 1 1 1 1 1 1 1 | PV<sub>max</sub> |
| PAM4365 | MexA::Tet | QPX7728     | 64  64 32 32 16 2 1 0.125 0.125 0.125 0.125 0.125 0.125 0.125 0.125 | 0.125 0.125 0.125 0.125 0.125 0.125 0.125 0.125 | 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 | 1 1 1 1 1 1 1 1 | PV<sub>max</sub> |
| PAM4126 | mxrF (MexAB-OprM)<sup>2</sup> | QPX7728 | 64  16 16 16 16 4 1 0.25 0.125 0.125 0.125 0.125 0.125 0.125 0.125 | 0.25 0.125 0.125 0.125 0.125 0.125 0.125 0.125 | 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 | 2 2 2 2 2 2 2 2 | PV<sub>max</sub> |
| PAM4129 | mxrE (MexCF-OprJ)<sup>2</sup> | QPX7728 | 8  8 4 4 0.5 0.06 0.06 0.06 0.06 0.06 0.06 0.06 0.06 | 0.06 0.06 0.06 0.06 0.06 0.06 0.06 0.06 | 0.03 0.03 0.03 0.03 0.03 0.03 0.03 0.03 | 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5 | PV<sub>max</sub> |
| PAM4132 | mxrE (MexEF-OprM)<sup>2</sup> | QPX7728 | 128 128 128 64 32 8 2 0.5 0.25 0.25 0.25 0.25 0.25 0.25 0.25 | 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25 | 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5 | 8 8 8 8 8 8 8 8 | PV<sub>max</sub> |
24

PAM2156 was obtained by transducing Pip-R marker from PAM1032 into PAM1020 using phage F116 as described in Methods section. The substitution in the AmpR protein.

L75R amino acid substitution in the

| Strain        | MexAB-OprM | AmpC1 | BL | Pip-R | Vaborbactam | Meropenem | Ceftolozane | Ceftazidime | Ceftazidime | Ceftazidime |
|---------------|------------|-------|----|-------|-------------|-----------|-------------|-------------|-------------|-------------|
| alone         | w/QPX at 4 µg/ml | w/QPX at 8 µg/ml | alone | w/QPX at 4 µg/ml | w/QPX at 8 µg/ml | alone | w/QPX at 4 µg/ml | w/QPX at 8 µg/ml | alone | w/QPX at 4 µg/ml | w/QPX at 8 µg/ml | alone | w/QPX at 4 µg/ml | w/QPX at 8 µg/ml |
| PAM1020 BL    | 1          | 0.5   | 0.5 | 0.5   | 0.5         | 0.5      | 0.5         | 0.5         | 0.5         | 0.5         | 4           | 4         | 4           | 4           |
| PAM1056 BL    | 489        | 2     | 0.5 | 0.5   | 0.5         | 0.5      | 8           | 0.5         | 32          | 1           | 1           | >256      | 16         | 4           | 4           |
| PAM1032 up    | 0.8        | 2     | 2   | 0.5   | 0.5         | 0.5      | 4           | 4           | 4           | 4           | 16          | 16        | 16          | 16          |
| PAM2156 up    | 769        | 4     | 2   | 2     | 0.5         | 1       | 16          | 4           | 4           | 4           | 64          | 8         | 8           | >256        |

All strains are derivatives of PAM1020 (PAM01). BL, basal level; up, overexpressed. Overexpression of the MexAB-OprM efflux pump in PAM1032 and PAM2005 is due to the L75R amino acid substitution in the MexR protein. PAM2005 was selected from PAM1032 on piperacillin at 64 µg/ml. It has AmpC overproduced due to the D159H amino acid substitution in the AmpR protein. PAM2156 was obtained by transducing Pip-R maker from PAM1032 into PAM1020 using phage F116 as described in Methods section. The

Biapenem MIC in the presence of BLIs at

NG, no growth

β between the MIC of the β-lactam antibiotics alone and in combination with QPX7728 (at 8 µg/ml) against isogenic strains of

P. aeruginosa

Specific efflux pump overexpressed due to mutations in respective regulators are shown in brackets

Mutation in mexZ also results in a partial downregulation of the carbapenem specific porin oprD

GM-MIC, a geometric mean of the antibiotic MIC values for the β-lactamase-producing and the vector only strain (calculated as the square root of the product of the antibiotic MIC values for the β-lactamase-producing and the vector only strain).

PV0.5, a concentration of a BLI to achieve 50% of antibiotic potentiation effect or a concentration of a BLI to reduce antibiotic MIC to or below the middle point of the MIC range between the MIC of the β-lactamase producing strain and the MIC of the vector only strain. The MIC middle point is a geometric mean of the antibiotic MIC values for the β-lactamase-producing and the vector only strain.

PVmax, minimal potentiating concentration of the BLI to reduce antibiotic MIC to the level seen in the parent strain that lacks KC (corresponding to complete inhibition of KC). NEL no growth.

Table 4: MIC (µg/ml) of various β-lactam antibiotics alone and in combination with QPX7728 (at 8 µg/ml) against isogenic strains of P. aeruginosa overexpressing MexAB-OprM and/or chromosomal AmpC

A geometric mean of the antibiotic MIC values for the β-lactamase-producing and the vector only strain (calculated as the square root of the product of the antibiotic MIC values for the β-lactamase-producing and the vector only strain).

PVmax, minimal potentiating concentration of the BLI to reduce antibiotic MIC to the level seen in the parent strain that lacks KC (corresponding to complete inhibition of KC). NEL no growth.

Biapenem MIC in the presence of BLIs at PV0.5 and PVmax are marked with yellow or green color, respectively.
numbers in the AmpC columns correspond to the expression of the chromosomal ampC relative to that in PAM1020. QPX. QPX7728. QPX7728 MIC against all strains is >128 µg/ml.

Table 5: Effect of varying concentrations of QPX7728 on biapenem MICs in isogenic OXA-23 producing strains of Acinetobacter baumannii overexpressing efflux pumps

| Strain  | Description          | Antibiotic | Antibiotic MIC (mg/ml) in the presence of varied QPX7728 (mg/ml) | MIC of the Recipient (µg/ml) | PVmax² (µg/ml) |
|---------|----------------------|------------|---------------------------------------------------------------|-----------------------------|---------------|
| ACM1565 | wild type            | Biapenem   | 32 32 16 2 1 0.06 0.016 0.125                                | 4                           |               |
| ACM1566 | AdeIJK overexpressed | Biapenem   | 16 16 4 2 0.5 0.06 0.06 0.125                                | 4                           |               |
| ACM1567 | AdeABC overexpressed | Biapenem   | 32 32 16 8 0.5 0.06 0.016 0.125                                | 4                           |               |
| ACM1566 | wild type            | Meropenem   | 32 32 16 4 0.25 0.03 0.5 4                                     | 4                           |               |
| ACM1566 | AdeIJK overexpressed | Meropenem   | 32 32 32 4 2 2 1 2                                        | 2                           |               |
| ACM1567 | AdeABC overexpressed | Meropenem   | 64 64 64 32 1 0.25 0.016 0.5                                   | 4                           |               |

¹ All strains produce plasmid encoded OXA-23. ACM1565, ACM1566 and ACM1567 were constructed by conjugating OXA-producing plasmid from the clinical strain AB1387 into rifampicin resistant derivatives of AB1007 (ACM1139, wild-type), ACM1027 (ACM1494, adeN mutant overexpressing AdeIJK), and ACM1030 (ACM1495, adeS mutant, overexpressing AdeABC), respectively.

²PVmax, minimal potentiating concentration of QPX7728 to reduce biapenem or meropenem MIC to the level seen in the parent strain that lacks OXA-23 (corresponding to complete inhibition of OXA-23). Biapenem and meropenem MIC in the presence of QPX7728 at PVmax are marked with green color.
Figure 1. QPX7728