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Engineering specificity from broad to narrow: Design of a BLIP variant that exclusively binds and detects KPC β -lactamase

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Abstract

The β -lactamase inhibitory protein (BLIP) binds and inhibits a wide range of class A β -lactamases including the TEM-1 β -lactamase (K_i = 0.5 nM), which is widely present in Gram-negative bacteria, and the KPC-2 β -lactamase (K_i = 1.2 nM), which hydrolyzes virtually all clinically useful β -lactam antibiotics. The extent to which the specificity of a protein that binds a broad range of targets can be modified to display narrow specificity was explored in this study by engineering BLIP to bind selectively to KPC-2 β -lactamase. A genetic screen for BLIP function in *Escherichia coli* was used to narrow the binding specificity of BLIP by identifying amino acid substitutions that retain affinity for KPC-2 while losing affinity for TEM-1 β -lactamase. The combination of single substitutions yielded the K74T:W112D BLIP variant, which was shown by inhibition assays to retain high affinity for KPC-2 with a K_i of 0.4 nM, while drastically losing affinity for TEM-1 with a K_i > 10 μ M. The K74T:W112D mutant therefore binds KPC-2 β -lactamase three times tighter while binding TEM-1 >20,000 fold weaker than wild-type BLIP. The K74T:W112D BLIP variant also exhibited low affinity (K_i > 10 μ M) for other class A β -lactamases. The high affinity and narrow specificity of BLIP K74T:W112D for KPC-2 β -lactamase suggests it could be a useful sensor for the presence of this enzyme in multi-drug resistant bacteria. This was demonstrated with an assay employing BLIP K74T:W112D conjugated to a bead to specifically pull-down and detect KPC-2 β -lactamase in lysates from clinical bacterial isolates containing multiple β -lactamases.

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Supporting Information

Supporting information is provided including a supplemental figure (S1) with representative time-course measurements to determine rate constants for BLIP mutants and β -lactamase as well as a figure (S2) showing SDS-PAGE analysis of the pull-down experiment of KPC-2 and TEM-1 by the K74T:W112D BLIP double mutant protein. This information is available free of charge via the Internet at https://urldefense.proofpoint.com/v2/url?u=http-3A__pubs.acs.org_&d=DQIFaQ&c=ZQs-KZ8oxEw0p81sqgiaRA&r=RfnpxYrj14iE1HuJ27NxYw&m=CQqQ5gLJy9muxac5HGtuacDnI9EFN3LJUbZ5JmXuwE8&s=5IFKxSWstmvuGtOyBMw7v8H0F64dtpiaT-hntYkQjRc&e=.

Keywords

protein-protein interactions; protein engineering; antibiotic resistance; β -lactamase; enzyme inhibition; diagnostics

Introduction

Protein-protein interactions mediate most cellular processes and the ability to modify these interactions represents a path to the development of new diagnostics and therapeutics. Engineering protein-protein interaction specificity is a challenging task that has been approached previously by experimental screening of libraries of mutants as well as by computational methods^{1–3}. Tailoring the specificity of an interaction often entails both positive and negative design strategies where positive design involves the introduction of substitutions in a protein that increase binding affinity with a target (A) and negative design involves introducing substitutions that result in decreased binding affinity for another target (B) such that the final engineered protein binds target A but not B^{4–7}.

The β -lactamase inhibitory protein (BLIP) is a 165 amino acid protein which binds and inhibits several class A β -lactamases including the TEM-1 and KPC-2 β -lactamases^{8–11}. BLIP is produced by the soil bacterium *Streptomyces clavuligerus*, which also produces the small molecule β -lactamase inhibitor clavulanic acid^{8, 12}. The interaction between BLIP and β -lactamases has been extensively studied as a model system to dissect the molecular determinants of protein-protein interactions. The fact that BLIP binds to a number of class A β -lactamases with a wide range of affinities (pM to μ M K_i 's) also makes it an interesting model for studying the determinants of specificity^{11, 13–15}. Previous studies on the molecular basis of specificity in the BLIP- β -lactamase system have identified several amino acid positions in BLIP that, when substituted, alter binding specificity^{11, 16, 17}. For example, the specificity of binding is significantly altered by mutation of two charged residues, E73 and K74, that are buried in the structure of the BLIP-TEM-1 complex¹⁸. In addition, substitutions of BLIP Y50, which resides on a loop that binds in the β -lactamase active site, also greatly alter binding specificity¹⁸, as do mutations in the residues W112 and R160¹⁷. Here we sought to extend this work by creating BLIP variants with very narrow binding specificity.

Antibiotic resistance among Gram-negative bacteria is an increasing problem and is a threat to the efficacy of antimicrobial therapy¹⁹. Resistance to the widely used β -lactam antibiotics is most often due to hydrolysis of the drugs by β -lactamase enzymes. The carbapenem class of β -lactam antibiotics is of value because of their broad spectrum of antibacterial activity and resistance to the action of β -lactamases²⁰. The widespread use of carbapenems, however, has resulted in the emergence of β -lactamases capable of hydrolyzing these antibiotics^{21, 22}. The KPC-2 β -lactamase hydrolyzes nearly all β -lactam antibiotics, including carbapenems²⁰. This enzyme emerged initially in *Klebsiella pneumoniae* strains but has spread to other Gram-negative species^{21–23}. The bacteria harboring KPC-2 β -lactamase often encode other resistance mechanisms, and strains resistant to nearly all antibiotics have been reported^{21, 24}. The early recognition of enzymes capable of

hydrolyzing carbapenem antibiotics among pathogenic bacteria is considered an essential step to treat infections and prevent the spread of the drug resistance gene²⁵.

BLIP is known to bind and inhibit TEM-1 β -lactamase with a K_i of 0.5 nM¹³. BLIP has also been shown to bind KPC-2 β -lactamase very tightly with a K_i of 82 pM and, therefore, it is a potential detection reagent for the enzyme⁹. However, to be a useful tool to distinguish KPC-2 β -lactamase from multiple other β -lactamases, it is necessary that BLIP bind exclusively to KPC-2. Here, we utilized a genetic screen to interrogate libraries of mutants focused at BLIP positions known to influence binding specificity in order to identify BLIP variants that can uniquely recognize KPC-2 β -lactamase without binding to other class A enzymes, including TEM-1. Combination of the single mutants yielded a variant with an over 25,000-fold change in specificity for binding KPC-2 versus TEM-1 and this variant also bound only weakly to other class A enzymes. The very narrow specificity of the mutant for KPC-2 suggests it could be used as a BLIP-based diagnostic tool to rapidly detect this β -lactamase from clinical isolates. The feasibility of using this approach was demonstrated by employing BLIP K74T:W112D conjugated to a bead to pull-down KPC-2 β -lactamase from crude lysates of laboratory and clinical bacterial isolates followed by detection with a chromogenic substrate.

Results

Protein engineering of BLIP binding specificity

The goal of this study was to create BLIP variants that bind to KPC-2 β -lactamase with high affinity without binding to other class A β -lactamases. For this purpose, a genetic screen for BLIP function was utilized to screen BLIP variants with amino acid substitutions in the binding interface for those that retained affinity for KPC-2 and simultaneously reduced affinity for other class A β -lactamases¹⁷. The genetic screen consists of inserting the gene for the β -lactamase of interest into the *pyrF* gene on the chromosome of *Escherichia coli* to maintain a low copy number and to introduce BLIP mutants into the strain on a plasmid¹⁷. Binding of the BLIP variant to the expressed β -lactamase reduces β -lactam antibiotic resistance levels of the strain, which can be readily detected by a decrease in the minimum inhibitory concentration (MIC) of ampicillin in an agar plate assay. We have previously shown that BLIP variants with high affinity for the TEM-1 β -lactamase result in low ampicillin MIC values for a strain with the TEM-1 gene inserted on the chromosome while strains containing weak binding BLIP variants exhibit high MIC values¹⁷. The genetic screen was extended for this study by inserting the gene for KPC-2 β -lactamase into the same site on the chromosome in the *pyrF* gene as the *bla*_{TEM-1} gene had been inserted previously using recombineering methods to create *E. coli* strain TP142.

In the first round of the genetic screen, BLIP mutants were introduced into *E. coli* TP142 encoding KPC-2 and the ampicillin MIC of the resulting clones was determined. The BLIP variants tested had been generated previously in a random mutagenesis study and consisted of mutants resulting from codon randomization of positions Y50, E73, K74, W112, and R160¹⁷. The positions were chosen based on previous results showing that substitutions at these sites alter binding specificity^{11, 16, 17}. The ampicillin MIC of the BLIP mutants was then tested in the *E. coli* strain containing the TEM-1 β -lactamase gene inserted into the

chromosome. Those mutants that exhibited a low ampicillin MIC on the KPC-2 strain (tight binding) and a high ampicillin MIC on the TEM-1 strain (weak binding) were chosen for further study. Four BLIP mutants, E73K, K74T, W112D and R160G, exhibited strong inhibition of KPC-2 and weaker inhibition of TEM-1 (Fig. 1). The MICs of these mutants in the TEM-1 strain are between 3 to 48 µg/ml (MIC for the TEM-1 strain with wild-type BLIP is 1 µg/ml and without BLIP is ~256 µg/ml), and in the KPC-2 strain they are less than 2 µg/ml (MIC for KPC-2 strain with wild-type BLIP is 1 µg/ml and without BLIP is 16 µg/mL).

The range of MICs for the mutants in the TEM-1 strain indicates that there is still significant inhibition of the TEM-1 enzyme by the mutants, suggesting that multiple substitutions are required to greatly decrease binding to TEM-1. Therefore, double mutant combinations of the E73K, K74T, W112D, and R160G single mutants were constructed and their specificity was assayed by determining the ampicillin MIC in the TEM-1 and KPC-2 *E. coli* strains. Four double mutants, E73K:K74T, K74T:W112D, E73K:R160G, and K74T:R160G, exhibited strong inhibition of KPC-2 (MICs < 3 µg/ml) and very weak to undetectable inhibition of TEM-1 β-lactamase (MICs > 256 µg/ml)(Fig. 1).

Biochemical characterization of the binding affinity of BLIP double mutants

The genetic screen identified BLIP variants that appear to have the desired binding specificity¹⁷. To confirm the achieved specificity and better understand the binding mechanism of these mutants, the BLIP single and double mutant variant proteins were expressed and purified for more detailed biochemical characterization. A spectrophotometric assay was used to measure BLIP inhibition of TEM-1 and KPC-2 β-lactamases in 50 mM phosphate buffer^{11, 13, 18}. Three BLIP single substitution mutants, E73K, K74T and R160G, exhibited higher affinity for KPC-2 versus TEM-1 as indicated by lower inhibition constants (Table 1). The W112D single mutant, however, exhibited weak inhibition of KPC-2 despite a low ampicillin MIC in *E. coli* TP142. One possibility for the apparent difference in affinity in *E. coli* versus the biochemical assay is a salt dependence for binding. Therefore, inhibition assays were also performed in 100 mM HEPES with 300 mM NaCl to evaluate possible effects of ionic strength on binding (Table 1). The salt concentration did not generally affect inhibition; however, the W112D single substitution mutant exhibited some salt dependence for inhibition of the KPC-2 β-lactamases with weaker inhibition at low salt concentrations. Nevertheless, the slightly increased potency of inhibition by the W112D mutant with increased salt does not explain the inhibition observed in *E. coli* as measured by MIC and it is currently unclear why the mutant displays weak binding in biochemical versus MIC experiments.

Among the four double mutant proteins examined, E73K:K74T and K74T:W112D bind to KPC-2 with at least nanomolar affinities or tighter, while E73K:R160G and K74T:R160G exhibit weaker than 100 nanomolar affinity (Table 2). Thus, in contrast to the BLIP W112D single mutant, the K74T:W112D double mutant exhibits potent inhibition both in the biochemical and the MIC agar plate assay. Binding kinetics measurements were also performed on the BLIP double mutants to determine the on and off rates for binding KPC-2 β-lactamase (Suppl. Fig. 1, Table 2). The results show that the BLIP double mutants have

similar association rate constants for binding KPC-2 β -lactamase, $\sim 4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, which is similar to other protein-protein interaction systems^{26, 27}. The two tight-binding double mutants, E73K:K74T and K74T:W112D, have dissociation rate constants of 4×10^{-4} and $3 \times 10^{-5} \text{ s}^{-1}$, respectively (Table 2). Because the association rates of the mutants are similar, the differences in the dissociation rate constants determine the differences in the affinities.

The four BLIP double mutants were also examined for inhibition of TEM-1 β -lactamase. The mutants did not exhibit significant inhibition of TEM-1 β -lactamase at up to micromolar concentrations. The BLIP K74T:W112D double mutant exhibits the tightest binding to KPC-2 while losing affinity for TEM-1 β -lactamase and was therefore examined further. Measurement of inhibition by the BLIP K74T:W112D double mutant to other class A β -lactamases including the SHV-1 and CTX-M-14 enzymes revealed inhibition constants at weaker than micromolar affinity (Fig. 2, Table 3). It was of interest to also examine the inhibition of the SME-1 β -lactamase. Like KPC-2, SME-1 is a class A carbapenemase enzyme and it has 55% amino acid sequence identity with KPC-2. Previous studies have shown that wild type BLIP inhibits SME-1 with a K_i of 2.4 nM¹⁸. The inhibition constant for BLIP K74T:W112D with SME-1 was found to be 450 nM, which corresponds to a 188-fold loss in potency compared to wild type BLIP (Fig. 2, Table 3).

The overall effect of introducing the double mutation, K74T:W112D into BLIP is to selectively enhance binding to KPC-2 (3-fold, Table 3) while significantly weakening BLIP's interaction with all of the other class A β -lactamases examined (Table 3). For example, the K_i for non-carbapenemase class A β -lactamases (TEM-1, SHV-1, CTX-M-14) is reduced to $>10 \mu\text{M}$. In the case of TEM-1, this amounts to a >20000 -fold reduction in affinity for the double mutant (Table 3). The K_i for inhibition of SHV-1 by wild-type BLIP is relatively weak ($K_i = 1.1 \mu\text{M}$) but nevertheless inhibition is at least 9-fold weaker for BLIP K74T:W112D. Similarly, wild-type BLIP exhibits a modest K_i of $0.8 \mu\text{M}$ for CTX-M-14 β -lactamase but the BLIP double mutant exhibits at least 12-fold weaker potency (Table 3). Finally, as described above, wild-type BLIP is a potent inhibitor of the class A carbapenemase SME-1 while the double mutant exhibits 188-fold weaker binding (Table 3). Therefore, the genetic screen to identify K74T and W112D single mutants and their combination into the K74T:W112D double mutant resulted in a narrow binding spectrum BLIP variant.

Detection of KPC-2 β -lactamase using the K74T:W112D BLIP variant

To examine the suitability of the BLIP K74T:W112D variant as a detection reagent and potential diagnostic tool, a pull-down experiment was performed with the K74T:W112D protein immobilized on a Talon metal-affinity resin via an interaction with the poly-histidine tag present on BLIP. The K74T:W112D protein was bound to Talon resin, washed, and aliquots of the resin were mixed with purified TEM-1 or KPC-2 β -lactamase. After an incubation to allow binding, the resin was washed to remove unbound β -lactamase. The intrinsic catalytic activity of the target β -lactamase to hydrolyze the chromogenic substrate nitrocefin was utilized to generate a positive signal for capture of the enzyme.

The KPC-2 enzyme activity was strongly inhibited in typical buffered solution due to the binding between BLIP and KPC-2. To dissociate the captured KPC-2 β -lactamase from the

BLIP K74T:W112D variant protein, a range of ethanol concentrations was added to the resin-bound complex. Experiments revealed that ethanol concentrations below 20% did not efficiently disrupt BLIP- β -lactamase binding while concentrations above 50% resulted in the inhibition of enzymatic activity, presumably due to protein denaturation. However, ethanol concentrations between 20 and 45% efficiently disrupted the BLIP- β -lactamase interaction and did not significantly inhibit the enzyme. Therefore, the addition of nitrocefin in 30% ethanol directly to the resin-bound complex allows detection of the KPC-2 β -lactamase without SDS-PAGE or immune-detection methods. Importantly, a visible change of the color change due to substrate hydrolysis was detected within 20 min. of addition of the nitrocefin-ethanol mixture. For the TEM-1 containing samples, no visible color change was detected even after overnight incubation with the nitrocefin-ethanol mixture, consistent with SDS-PAGE analysis, which showed the TEM-1 did not bind the BLIP K74T:W112D-resin.

The pull-down experiment was next performed with the K74T:W112D protein immobilized on a Talon metal-affinity resin with the addition of protein lysates from *E. coli* expressing either TEM-1 or KPC-2 β -lactamase (Fig. 3). The immobilized Talon resin/BLIP K74T:W112D/ β -lactamase complexes were washed and eluted by addition of the nitrocefin-ethanol mixture. As seen in Fig. 3, the KPC-2 enzyme was efficiently captured and detected by the BLIP K74T:W112D beads while TEM-1 β -lactamase was not. In addition, the hydrolysis of nitrocefin was not detected in lysates when the expression of KPC-2 β -lactamase was not induced (Fig. 3).

The BLIP K74T:W112D variant was also tested after covalent attachment to resins (NHS-activated agarose beads). Covalent immobilization provides advantages over the His-tag/Talon immobilization, such as stability of the complex and expansion to other detection methods. The covalently immobilized BLIP beads readily captured purified KPC-2 but not TEM-1 as indicated by SDS-PAGE analysis after elution of protein from K74T:W112D beads that were loaded with either KPC-2 or TEM-1 β -lactamase (Supplemental Fig. 2).

Detection of KPC-2 in clinical samples using BLIP K74T:W112D

The BLIP K74T:W112D resin pull-down assay was next tested using several clinical isolates of bacteria. Two isolates of *Klebsiella pneumoniae* from the collection of ATCC cataloged samples were used as controls: the KPC-2 β -lactamase containing strain BAA1705 as a positive control and the BAA1706 strain lacking KPC-2 as a negative control. The clinical isolates were grown in LB media overnight and 1 ml of culture was used to create a lysate (Materials and Methods). The bacterial lysates were mixed with BLIP K74T:W112D covalently immobilized on agarose beads for detection of β -lactamase using the nitrocefin-ethanol method. A total of 8 samples were randomly chosen from a pool of 20 clinical isolates that represented a collection of relatively diverse bacteria strains and infections. A number of the strains tested positive for KPC-2 β -lactamase in the pull-down-nitrocefin assay (Fig. 4A). All of the samples were also tested for the presence of the KPC-2 gene by PCR. It was found that all of the nitrocefin hydrolysis positive samples were also positive by PCR and, conversely, all of the nitrocefin hydrolysis-negative samples were negative by PCR (Fig. 4C). The samples were also tested for the presence of the TEM-1 β -lactamase gene by PCR and the samples that were TEM-1 positive, KPC-2 negative by PCR were also negative

for KPC-2 using the pull-down nitrocefin hydrolysis method. This confirms that the BLIP-based detection method is highly specific for the presence of KPC-2 β -lactamase in these clinical samples.

Discussion

Wild-type BLIP from *Streptomyces clavuligerus* is a broad-spectrum inhibitor of class A β -lactamases. In this study, a genetic screen for BLIP function in *E. coli* provided an effective means of sorting BLIP random mutants to identify variants that retained binding affinity towards KPC-2 while losing affinity towards TEM-1 β -lactamase and other class A enzymes. The genetic screen allowed for the use of both positive and negative design to alter binding specificity in that BLIP mutants could be rapidly tested for loss of binding and inhibition of TEM-1 β -lactamase (negative design) while retaining or enhancing affinity for the KPC-2 enzyme (positive design). The combination of positive and negative selections has been used in several systems to engineer binding specificity and selectivity including the colicin immunity family proteins, the LuxR transcriptional activator, and BH3 interactions with Bcl-family proteins ^{4, 5, 7}.

A relatively small number of single amino acid mutants were examined to find candidates that changed the binding specificity of BLIP. Randomization of only five positions in BLIP (Y50, E73, K74, W112, and R160) identified four single position substitutions that maintained binding to KPC-2 while losing affinity for TEM-1 β -lactamase. The high success rate for finding mutants that changed binding specificity is due to the choice of BLIP positions to interrogate, which was based on knowledge from previous studies that substitutions at these positions can change the binding specificity of BLIP ^{9, 11, 17, 28}. The strategy for engineering further changes in specificity was to make double mutant combinations of a subset of the single mutants with a focus on double mutants containing the E73K and K74T substitutions, which had the most significant changes in binding specificity as single substitutions. The energetic contributions to binding from a pair of mutated residues can be additive or the effects of the substitutions can be coupled due to energetic communication between the sites leading to non-additivity ^{1, 29, 30}. The effects of the four double mutants studied here on the binding constant for KPC-2 are largely non-additive with the exception of E73K:K74T. The most striking non-additive combination of BLIP substitutions is K74T:W112D. As described above, the W112D single mutant exhibited low ampicillin MIC values suggesting strong inhibition of KPC-2 β -lactamase; however, the biochemical assay with the purified mutant revealed weak inhibition. The combination of the W112D substitution with the K74T mutant exhibited strong inhibition of KPC-2 and very weak inhibition of TEM-1 both in the biochemical and MIC assays, revealing a large, non-additive effect for KPC-2 binding when measured using the inhibition assay. A detailed biophysical and energetic analysis of the combination of the mutations is beyond the scope of this report; nevertheless, the straightforward approach of combining individual substitutions with the desired binding properties resulted in the generation of a double mutant that binds tightly to KPC-2 β -lactamase but does not bind to other class A enzymes tested.

Biochemical characterization of the various double mutants with respect to affinity (K_i) as well as on and off-rates for binding KPC-2 β -lactamase revealed that the K74T:W112D double mutant possessed the highest affinity (0.4 nM K_i). A comparison of on and off-rates for BLIP mutants E73K:K74T, E73K:R160G and K74T:W112D binding to KPC-2 revealed similar on rates of $2\text{--}5 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ (Table 2). The higher affinity of BLIP K74T:W112D for KPC-2 β -lactamase compared to the other double mutants was due to a slower off-rate. The majority of the effect of a mutation on binding affinity being due to changes in off-rates is common and has been observed in other systems^{31, 32}. The specificity change of BLIP K74T:W112D for KPC-2 versus TEM-1 of more than 4 orders of magnitude ($>25,000$) represents one of largest specificity changes reported and is the result of only two substitutions^{2, 33–35}.

The possible molecular basis of the change in binding specificity of the BLIP K74T:W112D mutant was investigated by examining the structures of BLIP in complex with TEM-1 and KPC-2^{9, 10}. Because the BLIP K74 and W112 residues make direct contact with the helix loop region of class A β -lactamases, the sequences of the helix loop region of several class A enzymes were aligned as seen in Fig. 5. KPC-2 has a net charge of +1 along this loop (aa 99 – aa 114), while other class A enzymes, such as TEM-1 and SHV-1, have a net charge of –1. The double mutation K74T:W112D of BLIP will place a negative charge on top of this loop during binding. This electrostatic interaction (–1 from K74T:W112D vs +1 from the loop) is consistent with the K74T:W112D BLIP variant binding tightly to KPC-2 while binding weakly to other class A enzymes due to electrostatic repulsion (–1 from K74T:W112D vs –1 from the loop). A more detailed analysis explains some of the binding behavior of the single amino acid substitution mutants. The BLIP K74T substitution results in the loss of the salt bridge between TEM-1 E104/SHV-1 D104 and BLIP K74 in the TEM-1/BLIP or SHV-1/BLIP complexes, which would be expected to reduce binding affinity. In contrast, K74T eliminates the buried net charge in the region of W105 in the KPC-2/BLIP complex, which would be expected to retain or even increase the binding strength (Fig. 5). The W112D mutation would be expected to be unfavorable for binding all the class A enzymes due to charge repulsion with the conserved glutamic amino acid at position 110 (about 6 Å from BLIP W112). KPC-2, however, also has a lysine at position 99, which could attract the aspartate at position 112 in the W112D mutant and counteract the unfavorable interaction between E110 of KPC-2 and W112D of the BLIP mutant to some extent (Fig. 5).

A noteworthy observation is the effect of the electrostatic charge interactions across an interface on the stability of binding where the dominant binding forces are hydrophobic. Based on our studies with BLIP as well as other reported systems^{11, 32, 36–39}, an attractive interaction by opposite charges across an interface generally provides approximately 1.5–3 kcal of favorable energy^{11, 32, 36–39}. In addition, burying an unbalanced charge within an interface results in a relatively small unfavorable 1–2 kcal energy penalty^{28, 40} while engineering a charge-charge repulsion across an interface can elicit a large unfavorable energy penalty of more than 4 kcal as observed with the K74T:W112D variant in this study. These observations reveal that charge repulsion results in a larger destabilization energy than the stabilization energy provided by an electrostatic attraction. Understanding this behavior

of electrostatic interactions could help engineering specificity in protein-protein interactions such as using electrostatic collision to discriminate against unwanted interactions.

An interesting question is whether the results of the mutagenesis could be predicted computationally. To address this question the BLIP-TEM-1 (pdb id:1JTG) and BLIP-KPC-2 (pdb id:3E2K) were coordinates were submitted to the BeAtMusic server to predict changes in binding affinity due to the K74T and W112D substitutions ⁴¹. The BLIP K74T substitution was predicted to have a similar, modest effect on binding of both TEM-1 and KPC-2 (ΔG (kcal/mol) = 0.37 for TEM-1, 0.51 for KPC-2) while the BLIP W112D substitution was predicted to disrupt binding to both TEM-1 and KPC-2 (ΔG (kcal/mol) = 2.1 for TEM-1, 4.0 for KPC-2). Thus, the program did not predict that the substitutions would differentially affect binding to TEM-1 versus KPC-2. The BLIP K74T:W112D double mutant exhibits large non-additivity and was not tested computationally.

The binding specificity of the BLIP K74T:W112D double mutant provides the basis for a reagent to specifically detect KPC-2 β -lactamase. Biochemical characterization of the mutants and subsequent experiments confirmed the BLIP K74T:W112D variant can be used to capture KPC-2 specifically from clinical samples using a bead-based pull-down assay and when released with ethanol the captured enzyme has sufficient activity to hydrolyze the nitrocefin substrate giving a positive signal. The KPC-2 β -lactamase hydrolyzes nearly all β -lactam antibiotics, including carbapenems and therefore early detection of this enzyme could inform treatment strategies and prevent the spread of the drug resistance gene ²⁰. Bacterial clinical isolates often encode multiple β -lactamases, however, and so a detection reagent needs to recognize KPC-2 β -lactamase without binding other β -lactamases. As shown in this paper, the BLIP K74T:W112D double mutant has the necessary characteristics to specifically bind KPC-2 in the presence of other β -lactamases. The potential of this BLIP reagent as a diagnostic tool, however, will need to be evaluated with a much larger set of clinical isolates containing a variety of different class A β -lactamases. In addition, a number of natural variants of KPC β -lactamase with amino acid substitutions in or near the active site that result in a broadened substrate profile to include the extended-spectrum cephalosporin ceftazidime have emerged in recent years ^{42–45}. These variant enzymes are numbered consecutively and now include KPC-2 to KPC-17 ^{42–45}. Studies are in progress to test the ability of BLIP K74T:W112D to bind and detect these variants.

Methods

Bacterial strains and plasmids

The genetic screen for BLIP function was established by insertion of the *bla*_{TEM-1} into the *pyrF* gene of *E. coli* to create strain TP112 as described previously ¹⁷. The *bla*_{KPC-2} gene was inserted into the *E. coli pyrF* gene using similar methods with the recombineering strain *E. coli* SW102 whose genotype is: F- *mcrA* (*mrr-hsdRMS-mcrBC*) Φ 80d*lacZ* M15 *lacX74 deoR recA1 endA1 araD139* (*ara, leu*) 7649 *galU galK rspL nupG* [*lac*857 (*cro-bioA*) <> *tet*] ⁴⁶. The *bla*_{KPC-2} gene was introduced on a PCR product containing 35 base pairs (bp) of flanking sequence from the *pyrF* gene on each end. The primers used for amplification were:

pyrF-KPC-top-2

5'-CGACAAGATCGACCCACGCGATTGTCGTCTGAAGGTCGGCAAAG
AGATGATGTCACTGTATCGCCGTCTAGTTCTGCTGTCTTGTCTC-3'

pyrF-KPC-bot-2

5'-CACTGTCACAGCAATCAAAAGCGGTGCATCTTTGCCAAACGGAACC
AGTGCCCTCTTACTGCCCGTTGACGCCCAATCCCTCGAGCGCGAG-3'.

The *E. coli* SW102 strain was grown and concentrated for electroporation at 30°C. The *pyrF-blaKPC-2* PCR product was electroporated into *E. coli* SW102 and the cells were spread on agar plates containing 10 µg/ml ampicillin and 5 µg/ml tetracycline. The presence of the *blaKPC-2* gene on the chromosome inserted into the *pyrF* gene was validated by PCR amplification of the region followed by DNA sequencing. The genotype of the *E. coli* TP142 strain is: F- *mcrA* (*mrr-hsdRMS-mcrBC*) Φ 80d*lacZ* M15 *lacX74 deoR recA1 endA1 araD139* (*ara, leu*) 7649 *galU galK rpsL nupG pyrF::blaKPC-2* [*λc857 (cro-bioA)* <> *tetI*].

BLIP mutants were tested for function in *E. coli* TP112 (*pyrF::TEM-1*) and TP142 (*pyrF::KPC-2*) by transformation of the mutants in the context of the BLIP expression plasmid pGR32^{13, 17}. We had previously generated a collection of mutants derived from libraries randomized individually for codons Y50, E73, K74, W112 and R160, among others¹⁷. The collection of substitution mutants at each of these positions was further augmented by DNA sequencing of clones from each of the libraries. The collection of mutants included all 19 substitutions at Y50; V, R, W, N, A, S, Y, L, K, C, T, P, H substitutions at E73; Q, H, T, Y, L, I, A, S, V, R, G substitutions at K74; M, H, Y, L, I, K, P, A, S, V, R, D, G substitutions at W112; and H, T, F, L, P, A, S, N, G substitutions at R160. Each of these mutants was introduced into the *E. coli* TP112 and TP142 strains and the ampicillin MIC was measured using E-test strips. Those BLIP mutants that exhibited a low ampicillin MIC on TP142 (strong inhibition of KPC-2 β-lactamase) and an elevated ampicillin MIC on strain TP112 (weak inhibition of TEM-1 β-lactamase) were chosen for further study.

Construction of the BLIP double mutants

The E73K:K74T double mutant was constructed using the Quikchange oligonucleotide mutagenesis method with the primer blip73K-74T 5'-GTGGACTCGAAGAGCCAGAAAACCCTGCTGGCCCGAGCG-3'. The E73K:W112D, E73K:R160G, K74T:W112D, and K74T:R160G double mutants were constructed using either the E73K or K74T mutants as template DNA for Quikchange mutagenesis with primers introducing the W112D or R160G mutations. Similarly, the W112D:R160G mutant was constructed using the W112D mutant as template for mutagenesis with the R160G primer. All of the mutations were confirmed by DNA sequencing. The various double mutants in the context of the BLIP pGR32 expression plasmid were introduced into the *E. coli* TP112 and TP142 strains and ampicillin MICs were determined to evaluate the impact of the substitutions on BLIP binding to TEM-1 and KPC-2 β-lactamases.

Protein expression and purification

BLIP variant proteins were expressed from the BLIP expression plasmid pGR32 in *E. coli* strain RB791 using a prolonged induction strategy⁴⁷. The bacteria harboring a BLIP variant in the pGR32 plasmid were grown to log phase and induced with 6 mM of lactose for 28 to 30 hours at 23 °C, rather than the traditional 18 hrs. We observed that there was a large amount of bacterial lysis at the end of the induction, but the yield of BLIP was significantly increased. After induction, bacterial cells were harvested by centrifugation at 5,000 rpm for 15 min. and the culture media supernatant was retained. Bacterial pellets were resuspended in 20% sucrose, 10 mM Tris pH 8 and then added with 0.6 volume of ice cold water to osmotically shock the cells to release periplasmic trapped proteins. The osmotically released proteins were clarified by centrifugation at 15,000g x 30 min. These solutions were adjusted to final 150 mM NaCl and 10 mM imidazole. The culture media supernatant was concentrated and exchanged with TBS (10 mM Tris, pH 8, 150 mM NaCl, 10 mM imidazole) buffer with a tangential flow dialysis machine. This dialysis and buffer exchange step is needed to eliminate impurities that inhibit the Talon metal-affinity chromatography step. These solutions were passed through Talon cobalt columns. After washing, the bound proteins were eluted from the columns with 10 mM Tris, pH 8, 150 mM NaCl and 150 mM imidazole. These eluted proteins were further purified by a gel filtration chromatography step. There was no measurable binding potency difference between the BLIP purified from osmotic shock or from culture media. This expression and purification modification increases the BLIP yield significantly (at least 5-fold) compared to previous methods^{11, 17, 18}.

Determination of BLIP inhibition constants and binding rate constants

The β -lactamase inhibition assay was performed as previously described⁴⁸. Briefly, 1 nM of the enzyme was mixed with various concentrations of inhibitor proteins in 50 mM sodium phosphate, pH 7.0, and 100 μ g/mL of BSA for 15 min at room temperature. A second set of inhibition experiments were performed by mixing 1 nM enzyme with various concentrations of inhibitor proteins in 100 mM HEPES pH 7.0, 300 mM NaCl, 100 μ g/ml BSA for 15 min. at room temperature. For both sets of experiments, the amount of free enzyme was determined by monitoring initial hydrolysis rate of nitrocefin using OD at 483 nm at 23 °C. Nitrocefin was used at a concentration of 50 μ M for the inhibition assays. The K_M s for nitrocefin hydrolysis by the TEM-1 and KPC-2 β -lactamases in 50 mM sodium phosphate, pH 7.0, are 83 and 40 μ M, respectively, while the K_M s for nitrocefin hydrolysis by the TEM-1 and KPC-2 β -lactamases in 100 mM HEPES pH 7.0, 300 mM NaCl are 202 and 65 μ M, respectively. The K_i^{app} values for each BLIP mutant were determined by fitting the initial velocities to the Morrison tight-binding equation (Equation 1)⁴⁹:

$$E_{free} = [E_0] - \frac{[E_0] + [I_0] + K_i^{app} - \sqrt{([E_0] + [I_0] + K_i^{app})^2 - 4[E_0][I_0]}}{2} \quad (\text{Eq. 1})$$

where E_{free} is the concentration of free enzyme determined by residual activity of TEM-1 β -lactamase by comparison with the initial velocity of nitrocefin hydrolysis by the uninhibited

β -lactamase, $[E_0]$ is the total enzyme concentration and $[I_0]$ is the total inhibitor concentration.

Kinetic measurements of the association and dissociation of BLIPs with β -lactamases

Association and dissociation rate constants were measured using activity-based methods as previously described³². Briefly, 5 nM of KPC-2 or TEM-1 β -lactamase was mixed with 10 nM of a BLIP variant protein in 50 mM sodium phosphate pH 7.0 and 100 μ g/mL of BSA, and, after various designated time points, nitrocefin was added to the mixture to a final concentration of 150 μ M. The initial nitrocefin hydrolysis rate was measured using OD at 483 nm to determine the amount of active enzyme at that time point. The time-course of decreasing β -lactamase activity due to the binding of the BLIP variant protein can be fitted to following solution (Equation 2) of the second order kinetic equation 2

$$[ActiveEnz]_t = \frac{1 - B_1 \cdot B_4 - B_2 \frac{(e^{t \cdot C_1} \cdot C_2 + C_2 + e^{t \cdot C_1} - 1) \cdot C_1}{e^{t \cdot C_1} \cdot C_2 - C_2 + e^{t \cdot C_1} + 1}}{2 B_1} \quad (\text{Eq. 2})$$

where B_1, B_2, B_3, B_4, C_1 , and C_2 are intermediate variables defined as following

$$B_1 = k_{on}$$

$$B_2 = k_{off}$$

$$B_3 = ActiveEnz(0)$$

$$B_4 = BLIP - TotalEnz$$

$$B_5 = TotalEnz$$

$$C_1 = \sqrt{(4 \cdot B_2 \cdot B_5 \cdot B_1 + B_1^2 \cdot B_4^2 + 2 \cdot B_1 \cdot B_4 \cdot B_2 + B_2^2)}$$

$$C_2 = \frac{2 \cdot B_3 \cdot B_1 + B_1 \cdot B_4 + B_2}{C_1}$$

and where t is the time in seconds after the mixing, BLIP is the total BLIP concentration, $[BLIP]_t$ free BLIP at time t , TotalEnz is the total β -lactamase enzyme concentration, $[ActiveEnz]_t$ is the enzyme concentration at time t , ActiveEnz(0) is the initial free active enzyme, and $[complex]_t$ the inactive β -lactamase /BLIP complex at time t .

This function is a solution of the following second order kinetic equation

$$\frac{d[ActiveEnz]_t}{dt} = -k_{on}[BLIP]_t[ActiveEnz]_t + k_{off}[complex]_t \quad (\text{Eq. 3})$$

The experimentally determined time course of active enzyme after mixing with BLIP variant protein was fit to equation 1 to determine the k_{on} and k_{off} . This mixing approach can determine the k_{on} quite accurately but with a relatively large error in the k_{off} .

To determine the k_{off} more accurately, we diluted preformed β -lactamase/BLIP complex at least 100-fold in 50 mM Na phosphate, pH 7, 100 μ g/mL of BSA and 150 μ M nitrocefin to dissociate the complex to recover the active enzyme. After dilution, a time course of OD at 483 nm was recorded continuously in a Beckman Coulter DU 800 spectrophotometer. A time course of the recovery of the active enzyme was calculated based on the instantaneous slopes of the above OD time course using an extinction coefficient of 15000 $M^{-1} cm^{-1}$ and the enzyme's k_{cat} . This time course of the recovery of active enzyme was fitted to the above equation 1 to determine the k_{off} and the k_{on} . The method determines the k_{off} accurately but the k_{on} with a rather large error. Combining the mixing and dilution experiments, both association and dissociation rate constants were determined accurately.

β -lactamase pull-down experiments

To analyze and assess β -lactamase binding to the K74T:W112D BLIP variant protein in various conditions, pull-down experiments were performed using immobilized K74T:W112D BLIP protein. Covalently immobilized K74T:W112D BLIP was prepared using NHS-activated agarose resins (Thermo Scientific) according to manufacturer's instructions. These covalently immobilized K74T:W112D BLIP resins (15 μ L) were mixed with KPC-2 or TEM-1 in a 1 mL batch mode. After mixing in a cold room (6°C) for 1 hr, the resins were collected from nonbinding materials by brief centrifugation (5000 rpm for 5 min) and washed four times with TBS (10 mM Tris, pH 7.5, 150 mM NaCl) by spinning down the resins and suspending the resin pellet in 1.5 mL of fresh TBS. The resin pellets were mixed with 15 μ L of 10% SDS to elute the bound materials (final volume was 30 to 50 μ L). 10 μ L of the starting materials, nonbinding and bound materials were then analyzed by SDS-PAGE for protein binding.

To test for specific capture of KPC-2 β -lactamase from bacteria, cultures of bacteria harboring expression plasmids for KPC-2 or TEM-1 were induced with various concentrations of IPTG for 3 hrs at 37 °C. Bacteria were pelleted from 1.5 mL of the induced cultures by centrifugation at 10,000 g for 5 min. The bacterial pellets were suspended in 1 mL of 1% Triton-X 100 in TBS at room temperature for 30 min. to prepare the bacterial lysates. The lysates were clarified by centrifugation at 10,000 g for 20 min. to pellet the debris particulates. The clarified lysates (1 mL) were mixed with the 15 μ L of Talon immobilized BLIP K74T:W112D resins for 1 hr. in a cold room. These resins were collected by brief centrifugation and washed four times with TBS as above. The β -lactamases bound to the resins were assessed by addition of 100 μ M of the colorimetric substrate nitrocefin and 30% ethanol (final volume was ~30 to 50 μ L). The extent of nitrocefin hydrolysis was quantitated by the color intensity of samples in the tubes on the image. Quantitation was carried out using the ImageJ program. The color image was pasted into ImageJ and then split into three color channels. After inverting the green channel, the average pixel intensity was measured by a circular selection of the corresponding sample region on the image. The displayed average pixel values have been subtracted with a background value taken from a region on a tube.

Bacterial cultures from clinical samples were grown from inoculates overnight at 37°C. Bacterial lysates were prepared from these cultures similarly and mixed with 15 μ L of

covalently immobilized K74T:W112D resins for 1 hr. in a cold room. The resins were collected and washed as described above. The bound β -lactamases were assessed by the addition of 100 μ M of the chromogenic substrate nitrocefin and 30% ethanol (final vol. ~30 to 50 μ L).

PCR detection of *bla* genes

PCR detection of KPC-2 and TEM-1 was performed using primers 5'-CAAGCACAGCGAGGCCGTCAT and 5'-GCGGTAACCTACAGTTGCGCC for KPC-2, and 5'-CTATGTGGCGCGGTATTA and 5'-CCCAGTGCTGCAATGATA for TEM-1. The samples were first heat-treated at 95°C for 5 min. A total of 5 μ L of the heat-treated sample was added to a mixture containing KlenTaq polymerase, dNTPs, 1 μ M primers, and buffer to a final reaction volume of 50 μ L according to the manufacturer's instructions (Sigma). 35 cycles (1 min. 94°C denaturation, 30 seconds 50°C annealing, and 90 seconds 68°C extension) were carried out and the resulting products were analyzed and confirmed by 1% agarose gel electrophoresis to determine the existence of PCR products of the β -lactamase genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BLIP	β -lactamase inhibitory protein
k_{off}	dissociation rate constant
k_{on}	association rate constant
K_d	dissociation constant

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Figure 1. Summary of the steps taken to identify BLIP mutants with specificity for binding KPC-2 β -lactamase

In the first step, plasmid-encoded mutants from codon randomization of BLIP positions Y50, E73, K74, W112, and R160 were tested for ampicillin resistance levels in *E. coli* strains containing the gene for either KPC-2 or TEM-1 β -lactamase inserted in the chromosome. This screen identified four mutants with high MICs on the TEM-1 strain and low MICs on the KPC-2 strain (TEM-1/KPC-2 MICs in parentheses). The TEM-1 MICs of the single substitutions are less than 50 μ g/ml indicating significant binding is retained with TEM-1 β -lactamase. BLIP variants with increased selectivity for KPC-2 were engineered by constructing double mutant combinations of the four single substitutions identified in the initial screen.

Figure 2. Inhibition curve of β -lactamase activity versus concentration of BLIP K74T:W112D
Relative β -lactamase activity with nitrocefin as substrate is shown as a function of BLIP K74T:W112D concentration for various class A β -lactamases. Solid black circles represent KPC-2 β -lactamase and the solid line is the fitting curve giving a K_i of 0.4 nM. Open black circles represent SME-1 β -lactamase and the solid line is the fitting curve giving a K_i of 450 nM. Red squares represent TEM-1 β -lactamase activity while blue triangles indicate SHV-1 activity and green diamonds represent CTX-M-14 β -lactamase activity at various concentrations of BLIP K74T:W112D. Inhibition was not detected with the highest tested concentrations of BLIP K74T:W112D for the class A enzymes other than KPC-2 and SME-1. These results indicate that the K_i for TEM-1 and the other class A enzymes is significantly higher than 10 μ M.

Figure 3. Detection of KPC-2 β -lactamase using the BLIP K74T:W112D variant

The results of a pull-down experiment are shown for BLIP K74T:W112D bound to a cobalt Talon resin for detection of KPC-2 β -lactamase in a series of bacterial culture lysate samples. The TEM-1 and KPC-2 β -lactamase expressing *E. coli* cultures were induced and grown with the indicated concentrations of IPTG and bacterial lysates were obtained and bound to the BLIP K74T:W112D-resin. The bound β -lactamase was released with a mixture of nitrocefin (100 μ M) and ethanol (30%). The hydrolysis of nitrocefin by the released enzyme generates a yellow to red color change in the tube with the beads. The plot shows quantitation of the color intensity of samples in the tubes on the image. The numbers on the bars are the average pixel reading in the region of the corresponding regions on the image. Quantitation was carried out using the ImageJ program as described in Materials and Methods.

Figure 4. Detection of KPC-2 β -lactamase from clinical isolates of bacteria using the K74T:W112D BLIP variant

A. Detection of KPC-2 β -lactamase from bacterial lysates using BLIP K74T:W112D covalently immobilized to agarose beads. Strain BAA1706 is an ATCC strain of *Klebsiella pneumoniae* that does not contain KPC-2. ATCC strain BAA1705 is *K. pneumoniae* that contains KPC-2 β -lactamase. The other listed strains are clinical isolates. **B.** Quantitation of the color intensity of samples in the tubes on the image. The numbers on the bars are the average pixel reading in the region of the corresponding regions on the image. Quantitation was carried out using the ImageJ program as described in Materials and Methods. **C.** Comparison of detection results by BLIP K74T:W112D pull-down experiments and PCR detection of the *bla*_{KPC-2} gene in the samples.

Figure 5. Structural rationalization of the mutational effects of the BLIP K74T/W112D substitutions

Top panel. Sequence alignment of the helix loop regions (aa. 99 to 114) of the KPC-2 β -lactamase and the typical class A enzymes TEM-1 and SHV-1. Charged residues are indicated by color. Middle and bottom panels are structural depictions of residues W112 and K74 of BLIP in complex with KPC-2 (left panel from pdb code: 3E2K), with TEM-1 (middle panel from pdb code: 1TJG), and with SHV-1 (right panel from pdb code: 2G2U). For simplification, only the relevant structural features are presented. The helix loop regions (95 to 115 of class A enzymes) are drawn as space fill (middle panels) or tube (bottom panels) representations with the same view of the binding site in each panel. The W112 and K74 residues from BLIP are drawn as sticks. The relevant charged residues (residue E110 of class A enzymes, K99 of KPC-2, and D104 of SHV-1, E104 of TEM-1) are drawn as CPK representations. The relevant distances between residues are indicated by two-headed arrows and annotated with the distance.

Inhibition constants for purified single substitution BLIP variants for TEM-1 and KPC-2 β -lactamases.

Table 1

β -lactamase	BLIP wt and mutants					
	WT	E73K	K74T	W112D	R160G	
AMP MIC (TEM/KPC) ^a	(1/1)	(48/1.5)	(16/1.5)	(4/1)	(3/0.75)	
K_i for KPC-2 (nM)	1.2 $\pm$ 0.4 ^b	13.7 $\pm$ 6.6	1.8 $\pm$ 2.8	1084 $\pm$ 551	0.9 $\pm$ 0.4	
	0.2 $\pm$ 0.2 ^c	8.9 $\pm$ 6.3	1.5 $\pm$ 1.0	587 $\pm$ 63	0.4 $\pm$ 0.3	
K_i for TEM-1 (nM)	0.5 $\pm$ 0.4 ^a	835 $\pm$ 172	110 $\pm$ 23	1193 $\pm$ 701	236 $\pm$ 115	
	1.5 $\pm$ 0.6 ^b	2526 $\pm$ 578	151 $\pm$ 97	1266 $\pm$ 212	236 $\pm$ 85	

^a Ampicillin MIC (μ g/ml) for wild-type and mutants in *E. coli* strains encoding TEM-1 or KPC-2 with the MIC for the TEM strain to the left and KPC strain to the right.

^b Top row assays performed in 50 mM Na phosphate pH 7.0, 100 μ g /ml BSA, 50 μ M nitrocefin.

^c Bottom row assays performed in 100 mM HEPES pH 7.0, 300 mM NaCl, 100 μ g/ml BSA, 50 μ M nitrocefin.

Table 2

Inhibition constants and on and off rate constants for the BLIP double mutant variants for binding to KPC-2 β -lactamase.

BLIP mutant	K_i (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	k_{off}/k_{on} (nM)
E73K:K74T	1.6 ± 0.8	$3 \times 10^5 (\pm 25\%)$	$4 \times 10^{-4} (\pm 25\%)$	1.3
E73K:R160G	128 ± 30	$5 \times 10^5 (\pm 20\%)$	$3 \times 10^{-2} (\pm 25\%)$	60
K74T:W112D	0.4 ± 0.2	$2 \times 10^5 (\pm 30\%)$	$3 \times 10^{-5} (\pm 25\%)$	0.13
K74T:R160G	150 ± 57	Not determined	Not determined	N/A

Table 3Inhibition constants of wild-type and BLIP K74T:W112D for various β -lactamases.

β -lactamase	Wt BLIP K_i (nM)	K74T:W112D BLIP K_i (nM)	Fold Increase in K_i (K74T:W112D/wt BLIP)
KPC-2	1.2	0.4	0.3
TEM-1	0.5	>10000	>20000
SHV-1	1100	>10000	>9
CTX-M-14	810	>10000	>12
SME-1	2.4	450	188