Design of Potent β-Lactamase Inhibitors by Phage Display of β-Lactamase Inhibitory Protein*

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β-Lactamase inhibitory protein (BLIP) binds tightly to several β-lactamases including TEM-1 β-lactamase (Ki 0.1 nM). The TEM-1 β-lactamase/BLIP co-crystal structure indicates that two turn regions in BLIP insert into the active site of β-lactamase to block the binding of β-lactam antibiotics. Residues from each turn, Asp49 and Phe142, mimic interactions made by penicillin G when bound in the β-lactamase active site. Phage display was used to determine which residues within the turn regions of BLIP are critical for binding TEM-1 β-lactamase. The sequences of a set of functional mutants from each library indicated that a few sequence types were predominant. These BLIP mutants exhibited Ki values for β-lactamase inhibition ranging from 0.01 to 0.2 nM. The results indicate that even though BLIP is a potent inhibitor of TEM-1 β-lactamase, the wild-type sequence of the active site binding region is not optimal and that derivatives of BLIP that bind β-lactamase extremely tightly can be obtained. Importantly, all of the tight binding BLIP mutants have sequences that would be predicted theoretically to form turn structures.

β-Lactam antibiotics such as the penicillins and cephalosporins are among the most frequently used antibiotics. The major mechanism of bacterial resistance to β-lactam antibiotics is the production of β-lactamase. β-Lactamase catalyzes the hydrolysis of the amide bond in the β-lactam ring to create an ineffective antimicrobial (1). There are four classes (A–D) of β-lactamases based on primary sequence homology (2). TEM-1 β-lactamase is a class A enzyme and is the most prevalent β-lactam/β-lactamases including TEM-1 β-lactamase (Ki = 0.1 nM) (9, 10). The co-crystal structure of BLIP with TEM-1 β-lactamase indicates that a β-hairpin including residues 46–51 of BLIP inserts into the active site of β-lactamase (11). An aspartic acid residue at position 49 of the hairpin is positioned in the active site to form hydrogen bonds with four catalytic residues of β-lactamase. In addition, a phenylalanine at position 142 on the other loop occupies a position in the active site similar to the position that the benzyl group of β-lactam antibiotic penicillin G occupies during substrate binding and catalysis (11). Replacement of Asp49 with Ala lowers the binding affinity approximately 80-fold, whereas substitution of Phe142 with Ala results in a 300-fold decrease in binding affinity (10). Thus, Asp49 and Phe142 make important contributions to the stability of the inhibitory complex.

The display of proteins on the surface of filamentous phage is a powerful method to select variants of a protein with desired binding properties from large combinatorial libraries of mutants (12). The display of a protein of interest can often be achieved by fusing it to the N terminus of the M13 gene III coat protein (13, 14). High affinity variants can be obtained by direct selection of combinatorial libraries of mutant proteins displayed on the phage against a corresponding immobilized receptor protein (12, 14). Recently, we demonstrated that functional BLIP can be displayed on the surface of M13 phage as a fusion to the gene III coat protein (15). In this study we have created libraries of random mutants at positions including residues 46–51 of the β-hairpin of BLIP as well as within a region containing the important Phe142 residue. The libraries have been used to select BLIP derivatives that are potent inhibitors of TEM-1 β-lactamase.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Escherichia coli XL1-Blue (F−:Tn10 proA B lacIq ΔlacZAM15 recA1, endA1, gyrA96 (Nalr), thi-1, hsdR17, supE44, relA1, lac−) (16) (Stratagene, Inc.) was used for transfection of ligation reactions to construct the random libraries. E. coli TG1 (17) (F− traD36, lacIq, ΔlacZ M15 proA B lacIq Δ(hsdM rK B) ΔMCR1 (r− mcr− thi− Δ(lac proAB))) was used for production, amplification,

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1 The abbreviations used are: BLIP, β-lactamase inhibitory protein; PCR, polymerase chain reaction.
and determination of the titer of bacteriophage.

The pG3-BLIP vector encodes chromophenolamine resistance and contains BLIP as a fusion to the N terminus of the gene III coat protein (15). The secretion of the BLIP-gene III protein fusion is directed by the $\beta$-lactamase signal sequence, and transcription of the fusion is controlled by the constitutive $\beta$-lactamase promoter (15). For this study, the pG3-BLIP vector was altered by the insertion of a His$_6$ tag after the secretion signal sequence. The resulting vector, pTP154, directs the expression of BLIP with a His$_6$ tag at the N terminus to the surface of the phage. Both the pG3-BLIP and pTP154 vectors contain an amber codon between BLIP and gene III. Therefore, it is necessary to propagate phage from transformants containing an amber suppressor.

Library Construction—The BLIP 46–48, 49–51, and 141–143 random libraries were constructed by overlap extension PCR (18). The construction of the 46–48 library will be described as an example. The mutagenesis was accomplished with two rounds of PCR amplification with the following primers: BLIPXHOI, 5′-CGGGCGGCTCGAGGAGTAACAGGTCCCACTGCCGCT-3′; BLIPXBAI, 5′-CGGGGCCGTCTAGAATACAAGGTCCCACTGCCGCT-3′; and BLIPXHOI, 5′-CGGGGCCGAACNNSNNSNNSGACTACTACGCCTA-3′; L46–48-Top, 5′-GGGGGCACNNSNNSNNSGACTACTACGCCTA-3; and L46–48-Bot, 5′-GGGGGCACNNSNNSNNSGACTACTACGCCTA-3.

In the first round, an amplification was performed with the primers BLIPXHOI and L46–48-Top, and a separate amplification was performed with the primers BLIPXHOI and BLIPXBAI in a total volume of 100 ml for each reaction. In the second round, 1 ml of each of the two reactions from the first round was used as a template in an amplification using the BLIPXHOI and BLIPXBAI primers. The L46–48-Top and L46–48-Bot primers have complementary sequences, and therefore the PCR products from the first round have overlapping complementary sequences that allow an extension reaction to occur between the PCR products from round 1. The BLIPXHOI and BLIPXBAI primers serve to amplify the overlap extension products. The resulting PCR product was purified using a Qiaquick column (Qiagen) and digested with the restriction enzymes XhoI and XbaI. The digested DNA fragment was gel-purified and ligated with the pTP154 vector, which had been digested with SacI and XbaI. The ligation reaction was used to transform E. coli XL1-Blue cells by electroporation. A total of 2.9 × 10⁶ colonies was pooled for the 46–48 library, giving a >95% probability of all possible amino acid combinations represented in the libraries (19). The 49–51 and 141–143 libraries consisted of 3.9 × 10⁶ and 2.3 × 10⁵ pooled transformants, respectively.

Phage Preparation and Panning—E. coli cultures containing the pooled colonies from the library constructions were grown to $A_{600}$ of 0.6 in 25 ml of 2YT (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl per liter) supplemented with 12.5 mg/ml chloramphenicol. Approximately 1 × 10⁹ VCS M13 helper phage were then added, and the culture was grown overnight with shaking at 37 °C. The E. coli cells were removed by centrifugation, and the phage were precipitated from the supernatant with a 0.2 volume of 20% polyethylene glycol, 2.5 M NaCl. The phage were pelleted by centrifugation and resuspended in 0.01 original culture volume of STE (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA) (pH 8.0). The phage titer was determined by making serial dilutions of the phage displaying wild-type BLIP (data not shown).

BLIP and mutant BLIP derivatives contain a His$_6$ tag and were purified using metal affinity chromatography and gel filtration chromatography as described previously (10). The mutant BLIP genes were transferred from the phage display vector to the pTP123 expression vector by digestion with SacI and XbaI to release the BLIP gene followed by insertion of this fragment into pTP123 that had been digested with SacI and XbaI. The pTP123 expression vector provides for isopropyl-1-thio-b-D-galactopyranoside-inducible expression of BLIP (10).

BLIP Inhibition Assay—Inhibition assays were performed as described previously (10). The error listed in Table I is the error of the fit of nonlinear regression analysis to the curves shown in Fig. 5.

RESULTS

Selection of BLIP Variants from Combinatorial Libraries—Previously, we showed that functional BLIP can be displayed on the surface of M13 phage as a fusion to the gene III protein using a phagemid system (15). For the experiments described here the phagemid was modified to include a His$_6$ tag following the secretion signal sequence at the N terminus of mature BLIP. It is known that the addition of a His$_6$ tag to soluble BLIP does not affect its function (10). Enzyme-linked immunosorbent assay experiments confirmed that phage displaying the His$_6$-tagged BLIP have the same binding properties as phage displaying wild-type BLIP (data not shown).

BLIP and $\beta$-Lactamase Expression and Purification—Wild-type

$\beta$-Lactamase Expression and Purification—Wild-type

It is known from the crystal structure of the BLIP-$\beta$-lactamase complex as well as from mutagenesis results that the residues of the 46–51 $\beta$-hairpin as well as a second $\beta$-hairpin E. coli strains containing Phe$_{142}$ of BLIP make important interactions with the active site of TEM-1 $\beta$-lactamase (10, 11). Therefore, these design of potent $\beta$-lactamase inhibitors. BLIP residues were randomized in blocks of three to create libraries containing all possible amino acid combinations. BLIP residues were randomized in blocks of three to create libraries containing all possible amino acid combinations for positions 46–48, 49–51, and 141–143 (Fig. 1). Overlap extension PCR was used to create each of
rounds of panning. However, the closely related Pro 46-Ser47-
sequences, amino acid substitutions over the window shown. For the nucleotide for amino acid positions 46–48 were randomized to create all possible
Ser50-Tyr51 were observed, suggesting that this sequence en-
Pro46-Ser47-Asn48 enrichment was not due simply to chance,
set of panning experiments, the Pro 46-Ser47-Ala48 sequence
was again found. These results suggest that variants contain-
tional times. In the second set of panning experiments, the
was found. The number following the amino acid sequence is the number of
time the sequence was found in the population of clones sequenced.
the random libraries as described under “Experimental
Procedures” (18). All of the libraries contain greater than 10^6
codons and therefore have a >95% probability of containing all
codons a BLIP variant with tight binding affinity. The Asn49-
Ser47-Ala48, Asn 49-Gly50-Tyr51, and Gly 141-Ile142-Asn143 mu-
codes a BLIP variant with tight binding affinity. The Asn49-
Ser47-Tyr51 sequence was again detected among enriched
phage populations when the panning experiment was repeated
two additional times. The sequence Asn49-Gly50-Tyr51 was also
observed multiple times in the repeated panning experiments.
Among the clones obtained after three rounds of panning in
each of the trials, it is apparent that asparagine is strongly
preferred at position 49. This result is surprising because as-
partate at position 49 is thought to make critical interactions
with the active site of β-lactamase. A possible explanation is
that asparagine improves the stability of the type II’ turn and
that this improved stability compensates for altered interac-
tions with the active site.
Several types of sequences were selected after three rounds
of panning of the 141–143 library (Fig. 4). The strongest con-
sensus was observed for position 143, where asparagine was
present in 18 of the 29 clones that were sequenced. Large
hydrophobic residues similar to the wild-type phenylalanine
were observed at position 142 among the phage enriched for
binding. Previous studies indicate that substitution of the wild-
type phenylalanine with alanine at position 142 results in a
300-fold loss in binding affinity (10). These results suggest that
large hydrophobic residues can effectively substitute for phe-
lalanine at this position. At position 141 the wild-type gly-
mine and serine were the predominant sequences observed.
Taken together, the sequencing results for the three positions
suggest that the exact wild-type 141–143 sequence is not es-
tenial for binding.
Quantitation of BLIP-β-Lactamase Binding Affinity—The finding that many non-wild-type sequences were enriched after
panning of the BLIP libraries suggests that these mutants bind
β-lactamase as tightly as, or possibly more tightly than, does
wild-type BLIP. This hypothesis was tested by expressing and
purifying representative BLIP mutants from each of the librar-
ies. Each of the purified proteins was tested for the ability to
bind and inhibit purified TEM-1 β-lactamase in an in vitro
binding assay. The results of the binding assays for the Pro46-
Ser47-Ala48, Asn49-Gly50-Tyr51, and Gly141-Ile142-Asn143 mu-
tants are shown in Table I. These particular mutants were
chosen based on the frequency of occurrence among the se-
lected clones and on the ability to overexpress and purify these
proteins. It is apparent that the Pro46-Ser47-Ala48 mutant,
Despite having been selected from the library, binds β-lactamase with a similar affinity as does wild-type BLIP. In contrast, the Asn49-Gly50-Tyr51 mutant binds β-lactamase 10-fold tighter than does wild-type BLIP (Table I and Fig. 5). This result confirms that asparagine can effectively substitute for aspartate makes with the β-lactamase active site. The Gly141-Ile142-Asn143 substitutions do not act additively.

Combining BLIP Mutants to Test Additivity—Several studies have shown that mutations that individually increase binding affinity between proteins often act additively when combined into a single molecule (20, 21). This hypothesis was tested for the Asn49-Gly50-Tyr51 and Gly141-Ile142-Asn143 mutants by combining these substitutions into a single BLIP construct. The Asn49-Gly50-Tyr51/Gly141-Ile142-Asn143 BLIP mutant was purified and shown to inhibit TEM-1 β-lactamase with a Ki of 0.02 nM (Table I). Therefore, the Asn49-Gly50-Tyr51 and Gly141-Ile142-Asn143 substitutions do not act additively. This suggests that the sequence context of the 49–51 turn influences the interactions of the residues of the 141–143 turn with β-lactamase or vice versa.

**DISCUSSION**

The 46–51 BLIP turn structure containing Asp49 is a Type II′ β-turn (9). It is possible that amino acid substitutions within the turn in BLIP mutants that retain high binding affinity for β-lactamase maintain the Type II′ turn structure and simultaneously retain contacts with the β-lactamase active site. Alternatively, the substitutions may alter the conformation of the turn and promote new interactions with the β-lactamase active site. Hutchinson and Thornton (22) examined 3898 β-turns and derived sequence preferences for the i, i + 1, i + 2, and i + 3 positions that comprise a Type II′ turn. For the BLIP β-turn, Ala117 is at position i, Gly118 is at i + 1, Asp119 is at i + 2, and Tyr120 is at i + 3. According to the derived sequence potentials, tyrosine, valine, and serine are the preferred residue types at position i. The sequence replacement data obtained from the combinatorial phage display libraries indicate a strong preference for serine at position 47 (i). The fact that serine has a higher turn potential than the wild-type alanine has at the i position suggests that the serine substitution acts to stabilize the Type II′ turn structure.

At position i + 1, glycine has a much higher calculated turn potential than does any other residue (22). Glycine was also selected at position 48 multiple times from the 46–48 phage display library (Fig. 2). The next highest potentials for the i + 1 position are serine and alanine, which were also selected multiple times from the library (Fig. 2). Therefore, the results are again consistent with the notion that the substitutions selected from the libraries act to stabilize the Type II′ turn. It is also of interest that when a residue other than glycine occurs at position i + 1, a proline is almost invariably found at position 46, which is the i + 1–2 position (Fig. 2). A proline is also at this position in the leech decorin protein, which has an RGD motif in a Type II′ turn (23). The limited flexibility of proline may provide rigidity or stability to the turn that permits a residue other than glycine at position i + 1.

The residues with the highest calculated Type II′ turn potentials at the i + 2 position are asparagine followed by serine and then aspartate (22). Interestingly, asparagine was clearly the preferred residue at this position among the mutants se-
lected from the 49–51 phage display library (Fig. 3). Thus, a possible explanation for the observed preference for asparagine over aspartate at position 49 is that the Type II’ turn is more stable with asparagine at the i + 2 position and that this increased stability compensates for any interactions that are lost by replacement of the wild-type aspartate residue.

Threonine and glycine have the highest Type II’ turn potential at the i + 3 position (22). Glycine was repeatedly selected at this position from the 49–51 phage display library (Fig. 4). Thus, all of the Type II’ turn positions (47–50) the residues most frequently selected from the phage display libraries were those with high Type II’ turn potentials (22). This result suggests that stabilization of the Type II’ turn is an important factor in the selection for tight binding BLIP mutants from the random libraries. It is possible that a Type II’ turn evolved in this region because the sequence requirements for a Type II’ turn overlapped the chemical requirements for binding interactions of the BLIP side chains with the β-lactamase active site.

The predominant site of interaction between the turn containing residues 141–143 and β-lactamase is at phenylalanine 142 (11). The large, hydrophobic side chain is inserted in the β-lactamase active site where the chain makes several interactions (11). Consistent with an important role in binding, substitution of position 142 with alanine results in a 300-fold decrease in affinity (10). The results of the selection of binding mutants from the phage libraries suggest that the function of phenylalanine can be fulfilled by other large, hydrophobic side chains.

The Type II’ turn consisting of BLIP residues 47–50 is similar to a Type II’ turn in matrix proteins such as fibronectin that is used to bind cell surface integrins such as the platelet glycoprotein IIb/IIIa (24). These matrix proteins contain a consensus Arg-Gly-Asp (RGD) sequence within the Type II’ turn. The RGD sequence is found at the apex of solvent-accessible, flexible loops of proteins of different structural folds (24). The RGD Type II’ turn can be introduced onto other protein scaffolds where it retains binding function. In addition, cyclic peptides consisting of the RGD turn have been shown to retain the ability to bind integrins in the absence of the scaffold protein (25). The equivalent residues in BLIP to the Arg-Gly-Asp sequence are found in the Ala47-Gly48-Asp49 sequence. Studies are in progress to determine whether the 47–50 Type II’ turn of BLIP retains binding function when grafted onto other protein scaffolds and to determine whether a cyclic peptide containing the 47–50 region can interact with β-lactamase.

The results presented here indicate that although wild-type BLIP binds TEM-1 β-lactamase with a $K_i$ of 0.1 nM, the BLIP amino acid sequence is not optimally evolved for binding TEM-1 β-lactamase. This is not surprising because BLIP is produced by the soil bacterium S. clavuligerus and TEM-1 β-lactamase is found in enteric bacteria, and therefore these proteins did not co-evolve (8). However, the BLIP molecule must have evolved to bind a protein that closely resembles TEM-1 β-lactamase in order to coincidentally bind TEM-1 β-lactamase so tightly. The natural substrate of BLIP is unknown. Nevertheless, the finding that BLIP binds to multiple β-lactamases (9) and that the binding affinity can be adjusted by protein engineering presents an opportunity to design new inhibitors for these clinically important targets.

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