Protein Engineering vol. 16 no. 11 pp. 853–860, 2003
DOI: 10.1093/protein/gzg108

A broad-spectrum peptide inhibitor of β-lactamase identified using phage display and peptide arrays

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Hydrolysis of β-lactam antibiotics by β-lactamase enzymes is the most common mechanism of bacterial resistance to these agents. Several small-molecule, mechanism-based inhibitors of β-lactamases such as clavulanic acid are clinically available although resistance to these inhibitors has been increasing in bacterial populations. In addition, these inhibitors act only on class A β-lactamases. Here we utilized phage display to identify peptides that bind to the class A β-lactamase, TEM-1. The binding affinity of one of these peptides was further optimized by the synthesis of peptide arrays using SPOT synthesis technology. After two rounds of optimization, a linear 6-mer peptide with the sequence RRGHYY was obtained. A soluble version of this peptide was synthesized and found to inhibit TEM-1 β-lactamase with a K_i of 136 μM. Surprisingly, the peptide inhibits the class A *Bacillus anthracis* Bla1 β-lactamase with a K_i of 42 μM and the class C β-lactamase, P99, with a K_i of 140 μM, despite the fact that it was not optimized to bind these enzymes. This peptide may be a useful starting point for the design of non-β-lactam, broad-spectrum peptidomimetic inhibitors of β-lactamases.

Keywords: antibiotics/bacteriophage/inhibitors/peptide arrays

Introduction

The increased resistance of bacterial pathogens to clinically useful antibiotics has become a serious public health threat. It is therefore critical to identify new antimicrobials or to design inhibitors of antibiotic resistance-conferring enzymes. The β-lactam antibiotics such as the penicillins and cephalosporins are among the most often used antimicrobial agents. As with other antibiotics, resistance to these agents has been increasing in recent years. The most common mechanism of bacterial resistance to β-lactam antibiotics is the production of β-lactamases (Livermore, 1995). These enzymes are secreted by both Gram-positive and Gram-negative bacteria and provide resistance by catalyzing the hydrolysis of the β-lactam ring that is common to all antibiotics of this class.

β-Lactamases have been grouped into four classes based on primary sequence homology. Classes A, C and D are active-site serine enzymes that catalyze the hydrolysis of the β-lactam via a serine-bound acyl intermediate (Ghuysen, 1991). Class B enzymes require zinc for activity and catalysis does not proceed via a covalent intermediate (Carfi *et al*., 1995; Bush, 1998; Wang *et al*., 1999). The active-site serine β-lactamases belong to a larger family of penicillin-recognizing enzymes that includes the penicillin binding proteins (PBPs) that cross-link bacterial cell walls (Massova and Mobashery, 1998). The most prevalent plasmid encoded β-lactamase in Gram-negative bacteria is the class A TEM-1 β-lactamase that catalyzes the hydrolysis of both penicillins and cephalosporins (Frere *et al*., 1999). Extended-spectrum cephalosporins have been introduced in an effort to circumvent the action of class A β-lactamases such as TEM-1. The use of these agents, however, has resulted in the emergence of TEM mutant derivatives capable of hydrolyzing extended spectrum antibiotics (Petrosino *et al*., 1998).

An alternative method to combat β-lactamase-mediated resistance has been the use of mechanism-based, small-molecule inhibitors such as clavulanic acid and sulbactam (Bush, 2002). These inhibitors protect the β-lactam drug from hydrolysis by β-lactamases and restore the therapeutic potential of the antibiotic (Charnas and Knowles, 1981; Bush, 2002). Variants have now evolved, however, that resist these inhibitors while maintaining the ability to hydrolyze β-lactam antibiotics (Imtiaz *et al*., 1994; Henquell *et al*., 1995; Petrosino *et al*., 1998). Therefore, a need exists for the development of new inhibitors.

Phage display is a powerful technique for studying protein–ligand interactions [reviewed by Smith and Petrenko (Smith and Petrenko, 1997)]. The method involves the fusion of peptides or proteins to a coat protein of a filamentous bacteriophage (Smith, 1985). The peptides or proteins are normally fused to the N-terminus of the gene *III* phage protein. The gene *III* protein is a minor coat (3–5 copies per phage) protein located at the tip of the phage and is responsible for attachment of the phage to the bacterial F pilus in the course of the normal infection process (Rasched and Oberer, 1986). Because the gene encoding the fusion protein is packaged within the same phage particle, there is a direct link between the phenotype, i.e. the ligand-binding characteristics of a displayed peptide and the DNA sequence of the gene for the displayed peptide. This permits large libraries of peptides of random amino acid sequence to be rapidly screened for desired ligand binding properties (Smith and Petrenko, 1997).

Although phage display can be used to identify peptide ligands, these ligands generally do not bind the target protein with high affinity except in cases where the protein normally functions in peptide recognition (Clackson and Wells, 1994; Cochran, 2001). Peptide arrays offer a rapid means of optimizing the binding properties of peptides identified using phage display (Reineke *et al*., 2001; Reimer *et al*., 2002). The SPOT-synthesis method, for example, can be used to create large arrays of synthetic peptides on cellulose filters (Frank, 1992). The method employs Fmoc protection chemistry whereby the reagents are delivered automatically to discrete spots on the filters (Reineke *et al*., 2001). The resulting array

can be screened directly in the solid phase using an appropriately labeled target protein to identify peptides that bind the target with increased affinity (Reimer *et al*., 2002).

A combination of phage display and SPOT synthesis were used here to identify and optimize peptides that bind and inhibit TEM-1 β-lactamase. Surprisingly, the peptides optimized for binding the TEM-1 enzyme also inhibited the class A *Bacillus anthracis* Bla1 enzyme and the class C β-lactamase P99. These broad-spectrum peptide inhibitors may serve as the basis for the design of peptidomimetics that inhibit a wide range of β-lactamases.

Materials and methods

Phage display

The Ph.D.-C7C library (New England Biolabs) was purchased and used to identify peptides that mediated binding to immobilized TEM-1 β-lactamase. The Ph.D.-C7C library consists of random sequence 7-mers fused to a minor coat protein (pIII) of M13 phage. Biopanning was performed by coating a micro-plate well with 200 µl of purified TEM-1 β-lactamase at a concentration of 40 µg/ml in 0.1 M $NaHCO_3$ (pH 8.6) at 4°C overnight. The wells were then blocked with 200 µl of 5 mg/ml BSA in 0.1 M $NaHCO_3$ (pH 8.6), 0.02% NaN_3. After blocking, the C7C phage were input at 2×10^{11} pfu/well in 200 µl of wash buffer [1× TBS + 0.1% (v/v) Tween-20] and incubated at room temperature for 1 h. The wells were then washed 10 times with 200 µl of wash buffer to remove unbound phages and bound phages were eluted by the addition of 200 µl of 0.2 M glycine.HCl (pH 2.2) for 10 min at room temperature. The solution containing the eluted phages was neutralized by the addition of 25 µl of 1 M Tris, pH 8.0. The eluted phages were amplified by adding 150 µl of the neutralized solution to 25 ml of *Escherichia coli* ER2738 cells [F′ *proA+B+ lacI*^{q} Δ(*lacZ*)M15 *zzf*::*Tn10*(*Tet*^{R})/ *fhuA2 glnV* Δ(*lac-proA*B) *thi-1* Δ(*hsdS-mcrB*)5] (New England Biolabs) that had been grown to an OD_{600} of 0.2 and incubating the culture at 37°C for 4.5 h. The phages were precipitated by the addition of 1/5 volume of 20% PEG/2.5 M NaCl and harvested by centrifugation. The titer of the resultant phage stock was determined by infecting *E.coli* ER 2738 cells with serial dilutions of the stock and counting the number of resultant plaques. The second and third rounds of biopanning were performed using the amplified phage stocks from the previous round. The panning was identical except that the wash buffer for the second and third rounds was 1× TBS + 0.5% Tween-20.

DNA sequencing was performed to determine if the library was converging on a particular sequence. For this purpose, 20 single plaques were selected after the first round of panning and 40 plaques were selected following the third round of panning and single-stranded DNA was isolated and used as template for dideoxy DNA sequencing using the –96 sequencing primer (5′-CCCTCATAGTTAGCGTAACG-3′). The DNA sequencing reactions were performed with the ABI Big Dye Terminator Kit and the sequences were resolved using an ABI 3100 automated DNA sequencer.

Phage ELISA

Phage stocks for ELISA experiments were prepared by adding 5 µl of phage supernatant from the clone of interest to a 25 ml culture of *E.coli* ER2738 that had been grown to an OD_{600} of 0.1. The infected culture was then grown for 4.5 h at 37°C. The phages were harvested, precipitated and the titer was determined as described above. Wells of a micro-plate were coated with 20 µg/ml TEM-1 β-lactamase in 0.1 M $NaHCO_3$, pH 8.6 with 200 µl per well at 4°C overnight and blocked with 200 µl of blocking buffer at room temperature for 1 h. Serial dilutions of the phage stock were performed into wash buffer (1× TBS, 0.5% Tween-20) and 200 µl of each dilution were added to the coated wells. The wells were then washed six times with 200 µl of wash buffer. Phages that bound β-lactamase and were retained in the well were detected with an anti-M13 phage antibody conjugated to horseradish peroxidase (HRP) (Amersham). HRP was detected after the addition of the ABTS indicator reagent by monitoring the absorbance at 405 nm.

Synthesis and screening of cellulose-bound peptides

Cellulose-bound peptides were prepared by automated SPOT synthesis (Auto-Spot Robot ASP 222, Intavis, Bergisch Gladbach, Germany) as has been described in detail previously (Kramer *et al*., 1993, 1999; Wenschuh *et al*., 2000). Amino-functionalized membranes were purchased from Intavis. The membranes are derivatized with a polyethylene glycol spacer with a length of 8–10 ethylene glycol units (Intavis). The spacer also contains a free amino group to initiate peptide synthesis. Peptide synthesis was performed with Fmoc-protected amino acids as described previously (Wenschuh *et al*., 2000).

Two methods were used for the detection of β-lactamase binding to the SPOT membranes. The first method involved detection of bound TEM-1 β-lactamase using polyclonal anti-β-lactamase sera (data in Figure 3). For this experiment, the membrane was blocked overnight at 4°C with SuperBlock Buffer (Pierce) that was supplemented with 1 mg/ml bovine serum albumin (BSA) and 1 mg/ml casamino acids. The membrane was washed with 1× TBS containing 1 mg/ml BSA and 1 mg/ml casamino acids (wash buffer) and TEM-1 β-lactamase was incubated with the membrane at 0.1 µg/ml in wash buffer at room temperature for 1 h. The membranes were washed four times with wash buffer for 10 min each and a 1:10 000 dilution of rabbit anti-β-lactamase serum in wash buffer was incubated with the membrane for 1 h at room temperature. Detection was performed using a donkey anti-rabbit IgG conjugated to HRP. Bound antibody was detected using Amersham ECL chemiluminescent substrate and X-ray film.

For the second method, membranes were blocked overnight at 4°C with 1× TBS containing 1 mg/ml BSA and 1 mg/ml casamino acids. After washing with 1× TBS + 0.5% Tween-20, the membranes were incubated with 0.9 µg/ml TEM-1 β-lactamase conjugated to HRP for 2 h at room temperature. The membranes were then washed three times with 1× TBS–Tween-20 and bound β-lactamase-HRP was detected by chemiluminescence using X-ray film (ECL, Amersham) (data in Figure 4). Quantitation of the intensity of spots was performed by densitometry using a VersaDoc Imaging System (Bio-Rad).

Soluble peptide synthesis

All soluble peptides with the exception of the protein kinase substrates were prepared by solid-phase peptide synthesis using Fmoc-protected monomers at the Baylor College of Medicine protein chemistry core facility using an ABI 433A synthesizer. The HSACSDTRRGDCG-NH_2 peptide was cyclized by the dropwise addition of ammonium hydroxide to the solution to pH 8.0. The progress of the reaction was

monitored by reversed-phase high-performance liquid chromatography (RP-HPLC) and the final product was purified to >90% homogeneity by RP-HPLC. The HSAYSDTRRG-DYG-NH_2, RRGHYY-NH_2 and AAGHYY-NH_2 peptides were purified to >95% homogeneity by RP-HPLC. The identity of all synthesized peptides was verified by electrospray mass spectrometry at the Baylor College of Medicine protein chemistry core facility. The protein kinase substrates LRRASLG-NH_2 and RRKASGP were purchased from American Peptide Company (Sunnyvale, CA). These peptides were purified to >99% and were analyzed by mass spectrometry by American Peptide.

Purification of β-lactamase proteins

The TEM-1 β-lactamase was purified to >90% homogeneity using a zinc chelating Sepharose (fast flow) column (Pharmacia) and Sephadex G-75 gel filtration chromatography as described previously (Cantu *et al*., 1996). The P99, Bla1 and IMP-1 enzymes were expressed in *E.coli* and purified as described previously (Materon and Palzkill, 2001; Zhang *et al*., 2001; Materon *et al*., 2003).

β-Lactamase inhibition assays

Inhibition assays were performed as described previously (Petrosino *et al*., 1999; Rudgers *et al*., 2001). Briefly, various concentrations of peptide were incubated with TEM-1 (45 nM), Bla1 (0.9 nM), P99 (0.6 nM) or IMP-1 (0.8 nM) β-lactamase for 1 h in 50 mM phosphate buffer (pH 7.0) containing 1 mg/ml BSA. Following the incubation, the β-lactam substrate cephalosporin C (TEM-1, P99) or phenoxymethylpenicillin (Bla1) or nitrocefin (IMP-1) was added at a concentration at least 10-fold lower than the K_m of the substrate for the β-lactamase being tested. Hydrolysis of cephalosporin C was determined spectrophotometrically by measuring the decrease in A_{280} ($\Delta\varepsilon = 2390\ M^{-1}\ cm^{-1}$). Hydrolysis of phenoxymethylpenicillin (Pen V) by Bla1 was monitored by measuring the decrease in A_{240} ($\Delta\varepsilon = 570\ M^{-1}\ cm^{-1}$). Hydrolysis of nitrocefin was monitored by measuring the increase in A_{495} ($\Delta\varepsilon = 15\,000\ M^{-1}\ cm^{-1}$). The concentration of peptide that reduced enzyme velocity by half was the IC_{50} value. The K_i value was calculated from the IC_{50} using the method of Cheng and Prusoff (Cheng and Prusoff, 1973) using the equation $K_i = IC_{50}/(1 + [substrate]/K_M)$, where [substrate] is the concentration of β-lactam reporter substrate and K_M is the K_M value for the reporter for the enzyme being tested (Cheng and Prusoff, 1973). The data were fitted to determine the IC_{50} value using GraphPad Prism software.

The mode of inhibition of the RRGHYY-NH_2 peptide was determined using a Beckman DU-640 spectrophotometer in 50 mM potassium phosphate (pH 7) containing 1 mg/ml BSA in a final volume of 0.5 ml. All assays were performed in triplicate. Hydrolysis of cephalosporin C by TEM-1 and P99 and hydrolysis of Pen V by Bla1 was determined spectrophotometrically at 280 and 240 nm, respectively, using the molar absorbances given above. The peptide was allowed to incubate with TEM-1, P99 or Bla1 for 1 h prior to the addition of cephalosporin C (TEM-1, P99) or Pen V (Bla1). TEM-1 (45 nM) and P99 (5 nM) assays were carried out at 0, 50 and 100 μM RRGHYY-NH_2 and increasing concentrations of cephalosporin C. Bla1 (0.9 nM) assays were carried out at 0, 15, 30 μM RRGHYY-NH_2 and increasing concentrations of Pen V. K_m and V_{max} values were determined from double reciprocal plots at each concentration of RRGHYY-NH_2.

AGT	GAT	ACT	CGG	AGG	GGT	GAT	9/23
S	D	T	R	R	G	D	
AAG	CTT	GGT	CCG	ATT	CGG	GGT	5/23
K	L	G	P	I	R	G	
CTG	ACG	TCT	CAT	AAT	ATG	ATG	3/23
L	T	S	H	N	M	M	
CCT	TGG	ACG	AAG	GCT	TAT	CAT	3/23
P	W	T	K	A	Y	H	
AAT	TCT	TAT	TCG	CTG	TCT	CGG	2/23
N	S	Y	S	L	S	R	
CGC	TCT	CAT	ACT	CCG	CGT	AGT	1/23
R	S	H	T	P	R	S	

Fig. 1. Results of DNA sequencing of selected phage clones. Shown are the DNA and protein sequences of peptides selected after three rounds of biopanning on immobilized TEM-1 β-lactamase. The fractions to the right of the sequences indicate the number of times a particular sequence was identified out of the total of 23 clones sequenced.

Results

Selection of β-lactamase-binding peptides

The goal of these experiments was to perform an unbiased search to identify peptides from a random sequence library that would bind to the active site of TEM-1 β-lactamase. For this purpose, a phage display library displaying randomized 7-mer peptides flanked by a pair of cysteine residues was enriched for phage particles that bind to immobilized TEM-1 β-lactamase. After extensive washing to remove unbound phage, the bound phages were eluted from the immobilized β-lactamase and used to infect *E.coli*. The phages were amplified in the infected bacteria and used for another round of binding enrichment. After each round of binding enrichment, representative clones were randomly selected for DNA sequencing to determine if the library was converging on a particular sequence. After three rounds of panning, phages displaying six different peptide sequences were discovered (Figure 1). Among these, phages displaying the sequence CSDTRRGDC were predominant, suggesting that this is the optimal peptide ligand for β-lactamase binding.

Phage ELISA was used to verify that the CSDTRRGDC peptide identified by the phage display experiments bound to immobilized TEM-1 β-lactamase (Huang *et al*., 1998) (Figure 2). For these experiments, 10^{11} phages displaying the CSDTRRGDC peptide were added to a microtiter well that had been coated with 20 μg/ml TEM-1 β-lactamase. After extensive washing, bound phages were detected with an anti-M13 phage antibody. Phages displaying two additional sequences, CKLGPIRGC and CLTSHNMMC, were also assayed for binding (Figure 1). As a negative control, the phages were also tested for binding to the *E.coli* maltose binding protein (MBP). The results are consistent with the DNA sequencing results in that the CSDTRRGDC peptide exhibited the strongest binding to β-lactamase (Figure 2). Phages displaying each of the

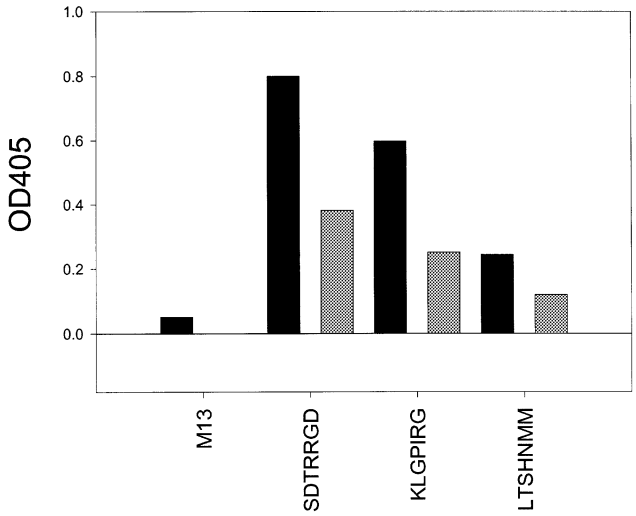

Fig. 2. Phage ELISA measurement of binding of selected phage to immobilized TEM-1 β-lactamase. Black bars indicate binding of phage to TEM-1 β-lactamase. Gray bars indicate binding of phage to *E.coli* MBP. The experimental error is <10% of the value in each case.

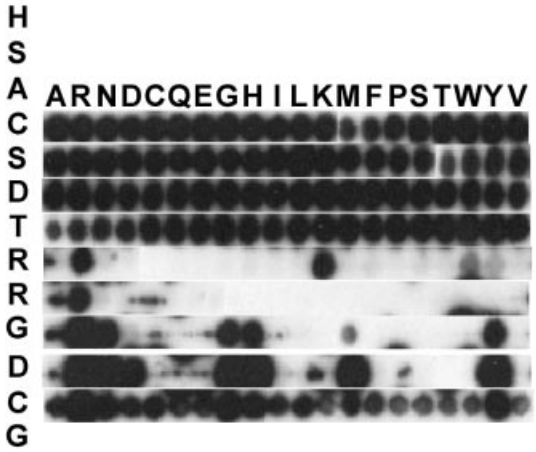

Fig. 3. Results of TEM-1 β-lactamase binding to peptides containing all single amino acid substitutions in the starting peptide HSACSDTRRGDCG. Each row represents the 20 amino acid substitutions at the listed position of the peptide. Note that the amino acid positions flanking the cysteines (**HSA**CSDTRRGDC**G**) were not substituted but are present in each substituted peptide on the array. Each column displays the indicated amino acid substitution at a particular position in the peptide.

peptides gave ELISA signals higher than background binding to MBP, suggesting that all three peptides bind specifically to TEM-1 β-lactamase.

Although the CSDTRRGDC peptide was selected based on the ability to bind TEM-1 β-lactamase, it is possible that the peptide could bind without inhibiting function. To establish if the peptide inhibits TEM-1 β-lactamase, the peptide HSACSDTRRGDCG-NH_2, which contains the 7-mer in addition to flanking sequences from the phage, was synthesized, oxidized and the disulfide-bonded version was purified. This peptide was used in a β-lactamase inhibition assay (Rudgers *et al*., 2001) and found to inhibit TEM-1 β-lactamase very weakly with a K_i of ~3.5 mM. Therefore, although the peptide bound and inhibited the enzyme, it clearly needed further optimization to be a viable inhibitor or lead peptide.

Table I. K_i determinations for inhibition of β-lactamases by peptides

Peptide	K_i (μM)		
	TEM-1	*B.anthracis* Bla1	*E.cloacae* P99
HSAYSDTRRGDYG-NH_2	298 ± 36	70 ± 8	254 ± 30
RRGHYY-NH_2	136 ± 20	42 ± 7	140 ± 13
AAGHYY-NH_2	438 ± 30	72 ± 6	468 ± 9
RR-NH_2	>2000[a]	>2000	>2000

[a]No inhibitory activity detected for this peptide against this enzyme up to 2 mM peptide concentration.

SPOT synthesis

The SPOT synthesis method can be used to synthesize large arrays of synthetic peptides on planar cellulose supports (Frank, 1992; Reineke *et al*., 2001). This technique was used to synthesize an array of peptides containing all possible single amino acid substitutions for the 7-mer sequence and the flanking cysteines in the HSACSDTRRGDCG peptide. The array was screened for peptides that bound TEM-1 β-lactamase by incubating the filter with purified, soluble TEM-1 β-lactamase. After extensive washing, β-lactamase that was retained on the filter was detected with an anti-β-lactamase polyclonal antibody in a format similar to western blotting (Figure 3). The results indicated that the SDT region of the C**SDT**RRGDC sequence did not contribute to binding since all substitutions function equally well at these positions. In contrast, the arginine residues appear to be critical for binding of the peptide to β-lactamase since substitutions at these positions eliminate binding (Figure 3). In addition, the disulfide bond, which constrains the peptide, does not appear important for binding. In fact, a substitution of the C-terminal cysteine with tyrosine appears to increase binding strength of the peptide to β-lactamase (Figure 3). Similarly, certain substitutions at the C-terminal glycine and aspartate positions of the CSDTRR**GD**C result in increased binding to β-lactamase.

In order to test whether substitution of the cysteine residues increases binding affinity as suggested by the array results, a soluble peptide was synthesized with the cysteines replaced by tyrosine residues. The HSAYSDTRRGDYG-NH_2 peptide was found to inhibit TEM-1 β-lactamase with a K_i of 298 μM (Table I). Therefore, replacement of the cysteine residues with tyrosines results in an ~10-fold increase in binding affinity, which is consistent with the qualitative result from the SPOT array.

Peptide binding to β-lactamase was optimized further by synthesis of another SPOT array. The previous array suggested that only the RRGDYG region of the peptide contributed to β-lactamase binding. In addition, the results of the previous array suggested that substitution of the aspartate residue (RRG**D**YG) with histidine to give a peptide with the sequence RRGHYG would result in improved binding. Therefore, the RRGHYG peptide and all single amino acid substitutions of this peptide were synthesized on the SPOT array (Figure 4). To ensure that the histidine substitution does in fact result in tighter binding, the RRGDYG peptide was also synthesized on the array for comparison. The array was probed with purified TEM-1 β-lactamase that had been conjugated to HRP, which allowed for direct detection of binding upon addition of the chemiluminescent HRP substrate. As predicted based on the results of the previous array, the RRGHYG peptide gave a stronger binding signal than the RRGDYG control peptide

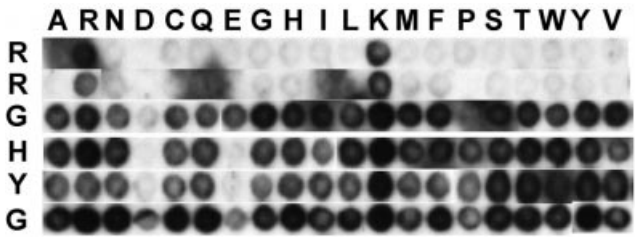

Fig. 4. Results of TEM-1 β-lactamase binding to peptides containing all single amino acid substitutions in the starting peptide RRGHYG. Each row represents the 20 amino acid substitutions at the listed position of the peptide. Each column displays the indicated amino acid substitution at a particular position in the peptide.

(Figure 4). The results also indicated that the two N-terminal arginine residues are important for binding to β-lactamase in that only arginine or lysine residues can substitute at these positions. In contrast, the glycine residue at position three (RR**G**HYG) can be substituted by all but negatively charged amino acids. Similarly, the histidine residue at position four (RRG**H**YG) can also be substituted by several different amino acids but not by negatively charged residues. Several different residues can substitute for the tyrosine at position five (RRGH**Y**G), although many of these substitutions, particularly negatively charged residues, result in less effective binding than the peptide containing tyrosine at this position. Finally, several amino acids, including tyrosine, when substituted for glycine at position six (RRGHY**G**) result in a more intense spot and presumably tighter binding. In summary, the SPOT synthesis results indicate a 6-mer peptide can bind to TEM-1 β-lactamase and that the N-terminal arginine residues are critical for this binding.

The soluble RRGHYY-NH_2 peptide was synthesized and tested for inhibition of TEM-1 β-lactamase to validate the SPOT synthesis results. This peptide includes the substitution of tyrosine for glycine at position six, which appears to contribute to tighter binding based on the array results (Figure 4). The peptide was found to inhibit TEM-1 β-lactamase with a K_i of 136 μM, which is an ~2-fold improvement in affinity relative to the HSAYSDTRRGDYG-NH_2 peptide and a 25-fold improvement relative to the disulfide-bonded HSACSDTRRGDCG-NH_2 peptide originally discovered by phage display (Table I).

The SPOT synthesis results suggest the arginine residues within the RRGHYY peptide play an important role in β-lactamase binding. This result was confirmed by determining the inhibition constant for the soluble peptide AAGHYY-NH_2. Substitution of the arginines with alanine residues resulted in a 3-fold increase in K_i for inhibition of TEM-1 β-lactamase, indicating that they contribute to inhibition (Table I).

Because the arginine residues are important for binding, the ability of soluble L-arginine to inhibit TEM-1 β-lactamase was assayed. Soluble L-arginine at concentrations up to 2.5 mM had no effect on β-lactamase activity, indicating that L-arginine is not an inhibitor of the enzyme. In order to address the possibility that the dipeptide Arg–Arg could inhibit the enzyme, an Arg–Arg-NH_2 peptide was tested and found to have no effect on β-lactamase activity at concentrations up to 2.0 mM. Therefore, although the Arg–Arg region of the peptide appears important for binding β-lactamase, it is not sufficient. It is possible, however, that the presence of the Arg–Arg motif in the context of a peptide of similar size to RRGHYY-NH_2 is sufficient for inhibiting β-lactamase. This possibility was tested using the commercially available protein kinase substrates LRRASLG-NH_2 and RRKASGP (Kemp *et al*., 1977; Pomerantz *et al*., 1977). It was found that these peptides had no effect on TEM-1 β-lactamase activity at concentrations up to 4 mM. Therefore, the specific sequence of the RRGHYY-NH_2 peptide appears important for inhibition.

Inhibition of other β-lactamases

Although the peptides were selected and optimized for binding to the TEM-1 β-lactamase of Gram-negative bacteria, it is possible these peptides may also inhibit other β-lactamases. Recently, the gene encoding the Bla1 class A β-lactamase from *B.anthracis* was cloned and the enzyme was expressed and purified from *E.coli* (Chen *et al*., 2003; Materon *et al*., 2003). The HSAYSDTRRGDYG-NH_2 and RRGHYY-NH_2 peptides were tested for inhibition of the *B.anthracis* Bla1 enzyme (Table I). Surprisingly, despite having been optimized to bind TEM-1 β-lactamase, each of the peptides was a more effective inhibitor of the Bla1 enzyme than the TEM-1 enzyme. For example, the RRGHYY-NH_2 peptide inhibits Bla1 with a K_i of 42 μM, which is ~3-fold lower than the K_i for inhibition of TEM-1 β-lactamase. Similarly, the HSAYSDTRRGDYG-NH_2 peptide inhibits Bla1 with a K_i of 70 μM, which is ~4-fold lower than the K_i for inhibition of TEM-1 β-lactamase. A possible explanation for the broad inhibition profile of these peptides is that the catalytic residues in the active site pocket of class A enzymes such as TEM-1 and Bla1 are highly conserved (Frere *et al*., 1999). Thus, although the enzymes are only about 30% identical, the active site pockets are very similar.

The peptides were also tested for inhibition of the class C β-lactamase, P99, from the Gram-negative bacterium *Enterobacter cloacae* (Lobkovsky *et al*., 1993; Dubus *et al*., 1996). The class A and class C enzymes have a similar fold and contain conserved amino acids that act similarly in catalysis (Lobkovsky *et al*., 1993). However, there are also many differences in the active site, which may explain why the mechanism-based inhibitors sulbactam and clavulanic acid are poor inhibitors of class C enzymes (Bush, 2002). Although the HSACDTRRGDCG-NH_2 peptide did not detectably inhibit P99 at concentrations up to 800 μM, the HSAYDTRRGDYG-NH_2 peptide inhibited P99 with a K_i of 254 μM and the RRGHYY-NH_2 peptide inhibited the enzyme with a K_i of 140 μM. Therefore, the RRGHYY-NH_2 peptide inhibits class A and class C enzymes with similar efficiency.

Because the RRGHYY-NH_2 peptide inhibited several enzymes, the specificity of these interactions was investigated further. The replacement of the arginines with alanines in the AAGHYY-NH_2 peptide resulted in a 3-fold increase in K_i for the P99 enzyme and a 2-fold increase for Bla1, indicating that the arginine residues are important for binding both of these enzymes (Table I). In addition, it was found that, similarly to the results with TEM-1, the dipeptide Arg–Arg-NH2 had no effect on the β-lactamase activity of these enzymes at concentrations up to 2.0 mM (Table I). These results suggest that the sequence requirements of the peptide for inhibition of the β-lactamases are similar. Because the arginine residues are important for inhibition, it is possible that a charge interaction contributes to binding. However, there is no correlation between binding and the p*I* of the enzyme in that the p*I* of TEM-1 is 5.4 whereas those of Bla1 and P99 are 9.1 and 8.7, respectively.

Finally, it was found that the RRGHYY-NH_2 peptide displayed no inhibition of the class B zinc-metallo-β-lactamase IMP-1 at concentrations up to 400 μM. This result is not surprising in that the zinc metallo-enzymes have a completely different fold and utilize zinc ions rather than a catalytic serine (Wang *et al*., 1999). Taken together, these results suggest the RRGHYY-NH_2 peptide is a broad-spectrum inhibitor of active-site serine β-lactamases and that this inhibition is dependent on the specific sequence of the peptide.

Mode of RRGHYY-NH_2 inhibition

In order to gain further insight into the mechanism by which the RRGHYY-NH_2 peptide inhibits β-lactamases, the inhibition patterns with respect to the TEM-1, Bla1 and P99 enzymes were determined. The RRGHYY-NH_2 peptide demonstrated a competitive inhibition pattern with respect to β-lactam substrates for each of the enzymes tested (Figure 5). This inhibition pattern suggests that the peptide acts similarly for each target by binding at or near the active site of the β-lactamases to block entry of the β-lactam substrate.

Discussion

Phage display has been shown to be an effective means of sorting large collections of random sequence peptides for variants that bind a target protein (Smith and Petrenko, 1997). Here we sorted a library of random 7-mers flanked by cysteine residues for peptides that could bind TEM-1 β-lactamase. From the panning results, a total of six different peptide sequences were identified. Of these selected peptides, the CSDTRRGDC sequence was identified multiple times and ELISA experiments confirmed that phage displaying this peptide bound to TEM-1 β-lactamase. A soluble peptide containing this sequence and also flanking sequence from the phage (HSACSDTRRGDCG-NH_2), however, inhibited β-lactamase very poorly.

SPOT synthesis was used to create peptide arrays to identify peptide variants that exhibit higher affinity for TEM-1 β-lactamase. SPOT synthesis permits rapid highly parallel synthesis of hundreds to thousands of peptides (Frank, 1992; Reineke *et al*., 2001). Furthermore, because the peptides are synthesized in an array format on a cellulose filter, it is straightforward to screen the array to identify peptides that bind a target protein. The method has been used extensively to map antigen–antibody interactions and other types of protein–protein interactions (Kramer *et al*., 1997; Knoblauch *et al*., 1999; Reineke *et al*., 2001). It has also been used to design a substrate-derived peptide that inhibits protein kinase G with a K_i of 7.5 μM and inhibits protein kinase A with a K_i of 750 μM (Dostmann *et al*., 1999). In our study, SPOT synthesis provided rapid information concerning which amino acids make the most critical contributions to binding β-lactamase. This information was used to design a peptide that binds TEM-1 β-lactamase with 25-fold higher affinity and is also reduced in size relative to the starting peptide identified by phage display.

In aqueous solution, peptides may assume multiple conformations, some of which may be favorable for binding to the target substrate. The lack of a fixed structure, however, for entropic reasons lowers the affinity of the peptide for the target protein (Cochran, 2001). The conformational flexibility of the peptide also obscures any connection between its solution properties and the active, bound conformation. Because of this, phage display libraries are often designed with cysteines flanking the peptide to be displayed (Wrighton *et al*., 1996). Because the gene III protein is secreted to the oxidative

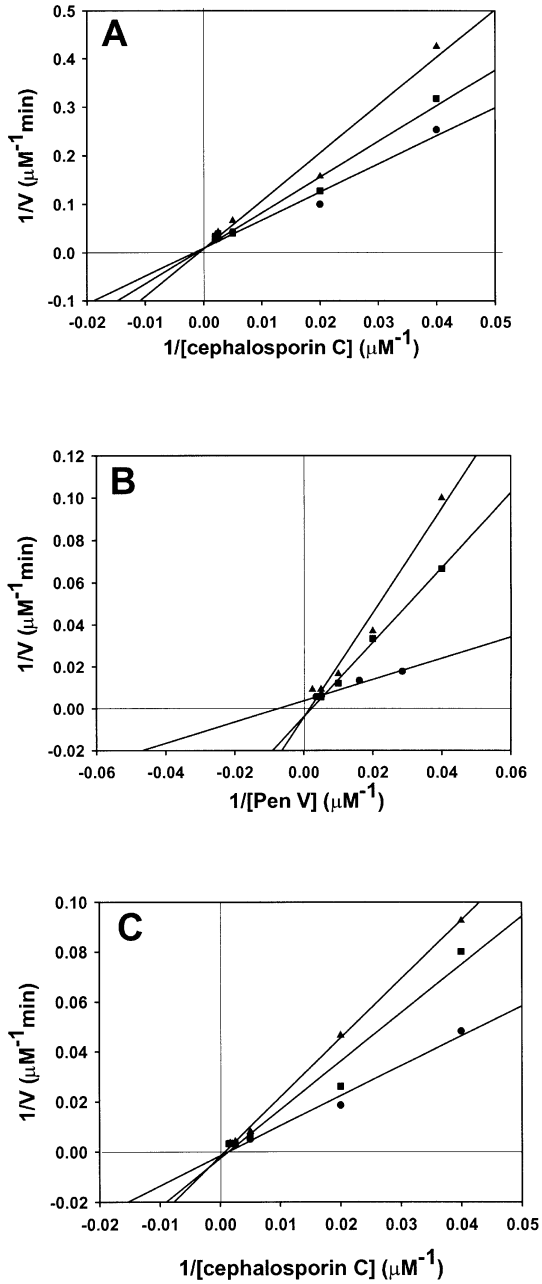

Fig. 5. Inhibition patterns of TEM-1, Bla1 and P99 β-lactamases by the RRGHYY-NH_2 peptide. (**A**) Inhibition of TEM-1 β-lactamase evaluated by determining the K_m and V_{max} for cephalosporin C hydrolysis with 0 μM (circles), 50 μM (squares) and 100 μM (triangles) RRGHYY-NH_2. Triangles, 100 μM RRGHYY-NH_2, K_m = 1179 ± 50 μM; V_{max} = 118 ± 12 μM/min. Squares, 50 μM RRGHYY-NH_2, K_m = 771 ± 31 μM; V_{max} = 105 ± 5 μM/min. Circles, 0 μM RRGHYY-NH_2, K_m = 598 ± 27 μM; V_{max} = 103 ± 6 μM/min. (**B**) Inhibition of Bla1 β-lactamase evaluated by determining the K_m and V_{max} for PenV hydrolysis with 0 μM (circles), 15 μM (squares) and 30 μM (triangles) RRGHYY-NH_2. Triangles, 30 μM RRGHYY-NH_2, K_m = 609 ± 14 μM; V_{max} = 243 ± 21 μM/min. Squares, 15 μM RRGHYY-NH_2, K_m = 450 ± 23 μM; V_{max} = 250 ± 11 μM/min. Circles, 0 μM RRGHYY-NH_2, K_m = 130 ± 19 μM; V_{max} = 256 ± 16 μM/min. (**C**) Inhibition of P99 β-lactamase evaluated by determining the K_m and V_{max} for cephalosporin C hydrolysis with 0 μM (circles), 50 μM (squares) and 100 μM (triangles) RRGHYY-NH_2. Triangles, 100 μM RRGHYY-NH_2, K_m = 1387 ± 88 μM; V_{max} = 630 ± 47 μM/min. Squares, 50 μM RRGHYY-NH_2, K_m = 981 ± 71 μM; V_{max} = 590 ± 64 μM/min. Circles, 0 μM RRGHYY-NH_2, K_m = 774 ± 42 μM; V_{max} = 674 ± 54 μM/min.

environment of the periplasm, cysteine residues within a single displayed peptide can be expected to form intrapeptide disulfides in at least a portion of the displayed peptides (Smith and Petrenko, 1997). A disulfide-constrained random 7-mer library was used here in an attempt to identify tight-binding peptides. Sequencing of clones selected by biopanning identified a peptide that was subsequently confirmed to bind immobilized β-lactamase by phage ELISA. The soluble peptide containing the disulfide bond, however, was a surprisingly poor inhibitor of TEM-1 β-lactamase with a K_i in the millimolar range. Synthesis of a peptide that replaced the cysteines with tyrosine residues bound much tighter, suggesting that the disulfide bond constrained the peptide in a conformation that was not optimal for binding. Why was this peptide selected as a binder in the phage display experiments? One possibility is that there is not a disulfide-constrained peptide in the library that is capable of binding tightly to β-lactamase and the selected peptide, albeit a weak binder, is nevertheless one of the best available in the library. Another possibility is that there is a mixture of oxidized and reduced peptides displayed on each phage particle and the binding of the reduced, linear peptide to the immobilized protein is responsible for the enrichment of the clone in biopanning. Regardless of the mechanism, it is clear that a disulfide-constrained version of a peptide is not necessarily the optimal binder.

An interesting property of the RRGHYY-NH_2 peptide is that, despite the fact that it was selected and optimized only for TEM-1 β-lactamase binding, it also inhibits the class *B.anthracis* Bla1 enzyme and the class C P99 β-lactamase. The identification of an inhibitor of the Bla1 enzyme is of particular significance in light of the threat of bioterrorism in addition to the agricultural implications that *B.anthracis* presents to livestock. Antibiotics of choice for the treatment of anthrax include tetracyclines (e.g. doxycycline), macrolides (e.g. erythromycin), quinolones (ciprofloxacin) and β-lactams (e.g. penicillin). Cavallo *et al.* tested 96 isolates of *B.anthracis* for their susceptibilities to 25 different antibiotics and found that 11.5% of the isolates conferred resistance to β-lactam antibiotics penicillin G and amoxicillin, most likely owing to the presence of a β-lactamase enzyme(s) (Cavallo *et al*., 2002). Further development of the RRGHYY-NH_2 peptide into a peptidomimetic could result in a useful inhibitor for the class A Bla1 enzyme. Because *B.anthracis* does not possess an outer membrane, a peptide-derived inhibitor would have access to secreted enzymes such as β-lactamase. In fact, with a molecular weight of 850, the RRGHYY-NH_2 peptide is already significantly smaller than vancomycin, a glycopeptide antibiotic commonly used to treat Gram-positive bacterial infections, which has a molecular weight of 1400 (Nieto and Perkins, 1971).

It is of interest that the RRGHYY-NH_2 peptide is 50% identical with a type II′ β-turn sequence found in the β-lactamase inhibitory protein (BLIP). BLIP is a 165 amino acid protein produced by *Streptomyces clavuligerus* that binds and inhibits TEM-1 β-lactamase with a K_i of 0.1–0.6 nM (Strynadka *et al*., 1994; Petrosino *et al*., 1999). The β-turn encompasses residues 46-AAGDYY-51 and was found in the co-crystal structure of the BLIP–β-lactamase complex to insert into the active site of β-lactamase (Strynadka *et al*., 1996). Based on this observation, a soluble peptide with the sequence CAAGDYYC was previously synthesized and found to inhibit TEM-1 β-lactamase with a K_i of 600 μM when oxidized and 490 μM when reduced (Rudgers *et al*., 2001). The 490 μM K_i value of the reduced CAAGDYYC is similar to the 438 μM K_i determined here for the AAGHYY-NH_2 peptide (Table I). Hence, it is possible that the RRGHYY-NH_2 peptide binds in a similar manner to the BLIP β-turn with the arginine residues providing increased affinity. This, in turn, suggests that the phage display and SPOT synthesis optimization methods converged on a similar β-lactamase-binding peptide to that which has evolved on BLIP.

An interesting question is how the RRGHYY-NH_2 peptide is capable of inhibiting three separate β-lactamases. The active site pockets of the TEM-1 and Bla1 enzymes are similar, hence many common interactions may occur between the peptide and these class A enzymes. The P99 β-lactamase, however, is a class C enzyme that possesses a similar overall fold to the class A enzymes but several differences in the active site region (Lobkovsky *et al*., 1993). The flexibility of the non-constrained RRGHYY-NH_2 peptide may contribute to the broad specificity. A non-constrained ligand can more easily adapt to altered binding sites than a conformationally constrained ligand (Ren *et al*., 2000). It is of interest in this regard that the BLIP-based CAAGDYYC peptide described above does not inhibit the P99 enzyme when oxidized but when reduced inhibits P99 with a K_i of 135 μM (Rudgers *et al*., 2001). This result supports a role for flexibility in binding. A detailed understanding of the mechanism of broad binding specificity, however, awaits structural information on the peptide–enzyme complexes.

Acknowledgements

We thank Amy Davidson for providing MBP and Hiram Gilbert for helpful advice and discussions. This work was supported by NIH grant AI32956 to T.P.

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Received June 25, 2003; revised September 3, 2003; accepted September 9, 2003