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Identification of peptide inhibitors of penicillinase using a phage display library

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ABSTRACT

There is a constant need to identify novel inhibitors to combat β-lactamase-mediated antibiotic resistance. In this study, we identify three penicillinase-binding peptides, P1 (DHIHRSYRGEFD), P2 (NIYTTPWGSNWS), and P3 (SHSLPASADLRR), using a phage display library. Surface plasmon resonance (SPR) is utilized for quantitative determination and comparison of the binding specificity of selected peptides to penicillinase. An SPR biosensor functionalized with P3-GGGC (SHSLPASADLRRGGGC) is developed for detection of penicillinase with excellent sensitivity (15.8 RU nM^{-1}) and binding affinity (K_D = 0.56 nM). To determine if peptides can be good inhibitors for penicillinase, these peptides are mixed with penicillinase and their inhibition efficiency is determined by measuring the hydrolysis of substrate penicillin G using UV–vis spectrophotometry. Peptide P2 (NIYTTPWGSNWS) is found to be a promising penicillinase inhibitor with a K_i of 9.22 μM and a K_i' of 33.12 μM, suggesting that the inhibition mechanism is a mixed pattern. This peptide inhibitor (P2) can be used as a lead compound to identify more potent small molecule inhibitors for penicillinase. This study offers a potential approach to both detection of β-lactamases and development of novel inhibitors of β-lactamases.

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Introduction

The synthesis of large numbers of antibiotics over the past few decades has caused complacency about the threat of bacterial resistance [1]. In recent years, increasing resistance of bacterial pathogens to clinically useful antibiotics has become a serious public health threat [2,3]. Among all antibiotics available nowadays, β-lactam antibiotics are still the most widely used and account for approximately 50–70% of total antibiotic use [4]. β-Lactam antibiotics include all antibiotic agents that contain β-lactam rings in their molecular structure. Typical β-lactam antibiotics include penicillins, cephalosporins, monobactams, and carbapenems [5]. Most β-lactam antibiotics work by irreversibly binding to penicillin-binding proteins (PBPs) to inhibit cross-linking of the peptidoglycan layer of bacterial cell walls, disrupting cell wall synthesis [6]. The primary cause of bacterial resistance to β-lactam antibiotics is the expression of β-lactamases, enzymes that attack and hydrolyze the β-lactam ring [7,8]. To combat β-lactamase-mediated antibiotic resistance, extended-spectrum β-lactam antibiotics have been introduced in an effort to circumvent the action of β-lactamases. However, the use of these agents has resulted in the emergence of mutants capable of hydrolyzing extended spectrum antibiotics [9]. An alternative method of combating β-lactamase-mediated resistance is the use of mechanism-based small molecule inhibitors. These inhibitors protect the β-lactam drug from hydrolysis by β-lactamases and restore the antibiotic effect. However, variants have now evolved that resist these inhibitors while maintaining the ability to hydrolyze β-lactam antibiotics [10,11]. Therefore, there is a constant need to find new β-lactamase inhibitors.

At present, small molecules such as clavulanic acid or clavulanate, sulbactam, and tazobactam are the mechanism-based β-lactamase inhibitors most commonly used to combat β-lactamase-mediated antibiotic resistance [12]. These molecules are often co-formulated with β-lactam antibiotics to combat microbial infection. Their IC_{50} (half maximal inhibitory concentration) values are in the range from 10^{-1}–10^{2} μM for serine-β-lactamase (class A, C, and D) [13]. However, metallo-β-lactamases (class B) are resistant to all mechanism-based inhibitors. They are only inhibited by thiols and chelating agents, and their IC_{50} values vary from low μM to mM [12]. Some protein and peptide inhibitors for β-lactamases were also studied in the past [14]. For instance, a β-lactamase inhibitory

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protein (BLIP) produced by *Streptomyces clavuligerus* was proved to be more effective than clavulanic acid against some class A β-lactamases [15]. Bounaga et al. described several cysteinyl peptides as competitive inhibitors of *Bacillus cereus* zinc β-lactamase, with inhibition dissociation constants in the 10^1–10^3 μM range [16]. Mandal et al. recently reported two novel five-amino-acids-long β-lactamase peptide inhibitors, which were tested in vivo in mice to neutralize bacterial resistance [17].

Phage display is a powerful selection technique for screening of novel peptides against a target molecule, in which a library of peptide variants is expressed on the outside of a phage virion, while the genetic material encoding each variant resides on the inside [18–20]. In the past, this technique has been used to identify peptide inhibitors for a broad range of enzymes [21]. Novel inhibitors have been isolated by screening phage display random peptide libraries on the immobilized enzymes, such as serine protease [22], trypsin [23], urease [24], β-glucosidase [25], transferase [26], ligase [27,28], and β-lactamase [29,30]. Huang et al. identified a peptide inhibitor of class A β-lactamase TEM-1 with a K_i of 136 μM. This peptide can also inhibit the class A Bla1 β-lactamase with a K_i of 42 μM and the class C P99 β-lactamase with a K_i of 140 μM, even though it was not optimized to bind these enzymes [29]. Sanschagrin et al. identified a peptide inhibitor of class B metallo-β-lactamase L-1 showing mixed inhibition with a K_i of 16 μM and K_i' of 9 μM [30]. Typically, enzyme-linked immunosorbent assays (ELISA) were used for qualitative determination of whether selected peptides bound to the target, without knowing the actual binding affinity between the selected peptides and the target [29,31,32]. Surface plasmon resonance (SPR) is a surface-sensitive optical technique that allows real-time and label-free measurements of biomolecular interactions in small volumes and high sensitivity [33–36]. Those advantages of SPR make it a better choice for studying the binding between peptides and the target than ELISA, and for detection of β-lactamases than conventional phenotypic and genotypic methods [37,38]. By using selected peptides from phage display as molecular receptors and SPR as the detection method, the binding specificity of selected peptides to the target can be quantitatively determined and compared.

Here, we aimed to identify peptide inhibitors of penicillinase from *B. cereus* for development of novel antimicrobials. Using a phage display library, we performed an unbiased search to identify peptides from a random sequence library that would bind to the penicillinase. SPR was utilized for determination and comparison of the specificity of binding selected peptides by phage display experiments to the penicillinase. However, not all peptides that bind to penicillinase are good inhibitors. Therefore, inhibitory activity of potential peptide inhibitors to the penicillinase were assessed in a functional assay in which hydrolysis of penicillin G by the penicillinase was monitored using UV–vis spectrophotometry. This study provides a tool for development of novel peptide inhibitors of β-lactamases. These peptide inhibitors may be a useful starting point for the design of novel small molecule inhibitors for β-lactamase after their binding mechanisms and structures are fully understood.

Materials and methods

Materials

Penicillinase from *B. cereus* lyophilized powder, L-cysteine (97%), penicillin G (≥98.0%), and potassium clavulanate were purchased from Sigma–Aldrich (Singapore). A Ph.D.-12 phage display peptide library kit was purchased from New England Biolabs (United States). Biacore gold sensor chips (untreated), borate buffer (10 mM disodium tetraborate pH 8.5 and 1 M NaCl), glycine hydrochloride (10 mM glycine hydrochloride, pH 2.0), and HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M sodium chloride, 3 mM EDTA, and 0.005% v/v surfactant P20) were purchased from GE Healthcare (Singapore). Peptides were synthesized by GenicBio Ltd. (Shanghai, China) with purity ≥95%. Deionized water (18 MΩ cm) was obtained from a Millipore filtration system.

Phage display

A Ph.D.-12 phage display peptide library (containing 1.0×10^{13} plaque-forming units (pfu) mL^{-1}, ~2.7×10^9 12-mer random peptide sequences) was used to identify peptides that bind to immobilized penicillinase from *B. cereus*. The phage display library screening was employed according to a standard protocol with some modifications [39]. (See the Supplementary Material for more details.) Briefly, the screening was carried out by incubating a library of random 12-mer peptides (displayed on phage) on a plate coated with penicillinase (target). After rigorous washing steps, only those phage-expressing peptides that bind to penicillinase were eluted. The eluted phage were then amplified in *Escherichia coli* and used in the next round of selection to enrich the binding peptide sequences (Fig. 1). After three rounds, individual clones were characterized by DNA sequencing. Peptide sequences binding to the target molecule were deduced from the phage DNA sequences.

Binding assays by SPR

To immobilize consensus peptides selected from phage display, a peptide linker, GGGC, was added to the C-terminus of these peptides. The peptides were synthesized and immobilized on gold surfaces through cysteine [39,40]. All SPR measurements were done using a Biacore T200 SPR system. First, peptide was immobilized on a bare gold sensor chip. Borate buffer (pH 8.5) containing 1.0 mg mL^{-1} of peptide flowed over the chip surface at 1 μL min^{-1} for 350 min. The surface density of immobilized peptide was determined using net increase of SPR response (for Biacore T200, a response of 1 RU is equivalent to 1 pg mm^{-2}) [41]. The sensor chip was flushed with HBS-EP buffer for more than 30 min to remove unreacted peptide. Next, the sensor chip surface was blocked with borate buffer containing 10 mM of cysteine, which is known to reduce nonspecific adsorption on the gold surface [36,42,43]. To test binding of penicillinase to the immobilized peptide, HBS-EP buffer containing penicillinase was injected at 2 μL min^{-1} over a period of 3 min. After the injection, the flow cell was flushed with fresh HBS-EP buffer for 3.5 min to remove unbound penicillinase. The binding level of penicillinase was determined by measuring the

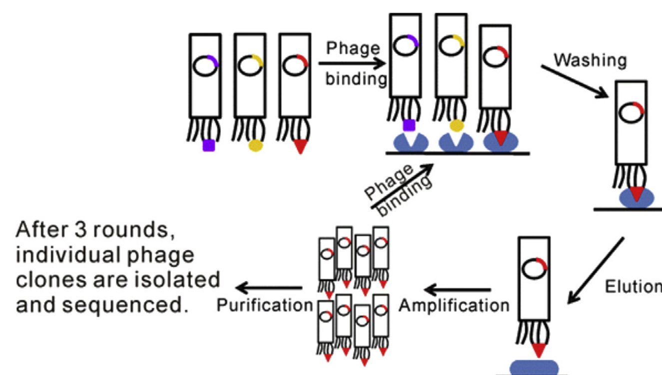

Fig.1. Schematic illustration of using a phage display peptide library for selection of penicillinase-binding peptides.

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