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A Novel Strategy for In Vitro Selection of Peptide-Drug Conjugates

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Summary

The chemical diversity of peptide and protein libraries generated from biological display systems is typically confined to the 20 naturally occurring amino acids. Here, we have developed a general strategy to introduce non-natural side chains into mRNA-display libraries via specific chemical derivatization. We constructed a mRNA-display library containing 3×10^{12} different peptides bearing a pendant penicillin moiety in a fixed position. In vitro selection using this hybrid peptide-drug library resulted in novel inhibitors of the *Staphylococcus aureus* penicillin binding protein 2a (PBP2a). This strategy resulted in a penicillin-peptide conjugate that has at least 100-fold higher activity than the parent penicillin itself. Our approach provides a convenient way to enhance the efficacy of known drugs and facilitates the discovery of powerful new hybrid ligands with functionalities beyond those provided by the 20 naturally occurring residues.

Introduction

Combinatorial peptide libraries are rich reservoirs for discovery of novel ligands against therapeutically interesting targets, including agonists [1] or antagonists of receptors [2], epitopes of antibodies [3], and inhibitors of enzymes [4]. Techniques such as phage display [5, 6], ribosome display [7, 8], and mRNA display [9, 10] can generate peptides that are physically associated with their own genes, making it feasible to identify molecules with desired properties through iterative cycles of enrichment and amplification. Because these libraries are made in biological systems, the chemical diversity is generally restricted to the 20 naturally occurring amino acids. By comparison, synthetic peptide libraries can contain numerous nonstandard residues but typically represent much smaller sequence complexity than display approaches. Additionally, identification of active molecules in synthetic libraries often requires complex deconvolution [11] or sophisticated encoding strategies [12]. We have therefore been very interested in developing approaches that allow nonnatural residues to be incorporated into highly complex natural display libraries.

The mRNA display approach allows libraries containing more than 10^{13} independent peptides or proteins to be constructed entirely in vitro, providing the highest sequence diversity currently available with any method [13, 14]. Additionally, because all steps in the mRNA display process are conducted in vitro, a variety of strategies are possible, such as in vitro mutagenesis [15], recombination [16], and nonsense suppression [17], that are not easily available to approaches such as phage display [6] or the yeast two-hybrid systems [18].

β-lactam drugs are antibiotics widely used to treat bacterial infections and function as irreversible inhibitors of the penicillin binding proteins (PBP) required for bacterial cell wall synthesis [19]. Since the introduction of these drugs, numerous bacterial strains have gained resistance against them using a variety of mechanisms. One of the most common modes of resistance is conferred by β-lactamase, an enzyme which hydrolyzes these drugs by opening the lactam ring [20]. Clinically, a variety of strategies have been pursued including the use of irreversible β-lactamase inhibitors [21] that are coadministrated with the drug. For example, Augmentin is a broad spectrum antibiotic that consists of a mixture of ampicillin and the irreversible β-lactamase inhibitor clavulanic acid. However, these drugs can be useless to treat infections resulting from several strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [22], where resistance is conferred by PBP2a, a penicillin binding protein that is sufficient for cell wall synthesis even at drug concentrations where other penicillin binding proteins are covalently inactivated [23–26]. Strains such as MRSA impose a serious health threat and necessitate the development of more powerful antibiotics.

We were interested in using mRNA display to isolate peptide-penicillin conjugates that could function against PBP2a, the primary β-lactam-resistance determinant in MRSA. Previously, we had used nonsense suppression to insert the nonnatural residue biocytin into an mRNA display library and select for its function [17]. Here, we demonstrate an alternative strategy incorporating a penicillin side chain into an mRNA display library via chemical derivatization and selecting for functional drug-peptide conjugates. This approach has allowed us to identify a series of novel penicillin derivatives (mol wt ~1500; cf. vancomycin, mol wt = 1449 g/mol) that inhibit *Staphylococcus aureus* PBP2a.

Results and Discussion

Constructing a mRNA Display Library Containing a Penicillin Side Chain

We began by working to append penicillin to a mRNA display library. Previous work with penicillins indicated that numerous modifications have been made to the 6 position of the β-lactam ring to enhance the pharmacokinetics and other properties of β-lactam drugs [27]. We therefore constructed the cysteine-reactive penicillin derivative 6-bromoacetyl penicillanate (Figure 1A) to allow the chemical diversity present in our peptide libraries to be displayed in a similar fashion.

A natural mRNA display peptide library containing the sequence X_5CX_5, constructed essentially as described [28], was used to generate the hybrid drug-peptide li-

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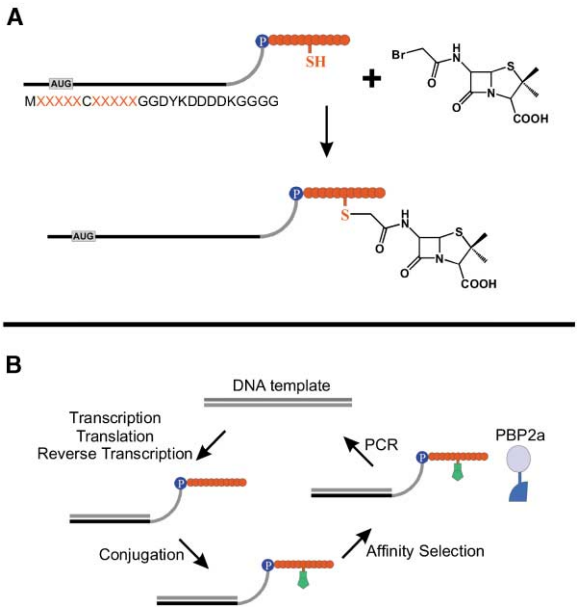

Figure 1. Library Construction and Selection Scheme

(A) Construction of an mRNA-display library containing penicillin. A fixed cysteine residue in the mRNA fusion library (–X_5CysX_5–) reacts with sodium 6-bromoacetyl penicillanate to form a peptide-drug conjugate.

(B) In vitro selection cycle. A double-stranded PCR DNA library is constructed, transcribed into mRNA, ligated with an oligonucleotide bearing a 3′ puromycin (P), and translated in vitro to form the mRNA-peptide fusion product. Reverse transcription and reaction with bromoacetyl penicillin yields a cDNA/mRNA-peptide library bearing the drug appended to the fixed cysteine. After reaction with PBP2a, functional molecules are eluted with DTT, and the template is amplified by PCR for use in the next selection cycle.

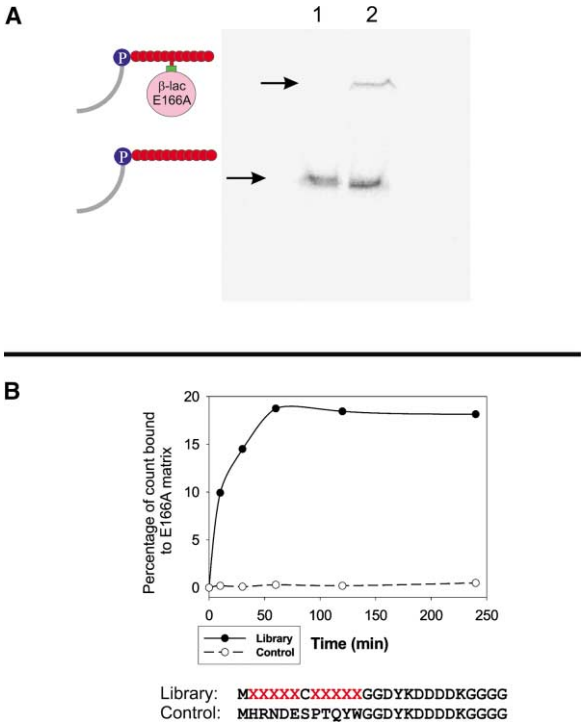

Figure 2. Efficiency and Specificity of the mRNA-Display-Penicillin Coupling Reaction

(A) Electrophoretic analysis. Prior to treatment with sodium 6-bromacetyl penicillanate, the RNase-treated mRNA-display library shows no interaction when (E166A) β-lactamase is added (lane 1) ([^{35}S]cysteine label). After coupling, approximately 20% of the RNase-treated library moves with lower mobility, consistent with attachment of the 30 kDa mutant lactamase (lane 2).

(B) Specificity of library modification. The X_5CX_5 library and a control template lacking cysteine were both reacted with sodium 6-bromacetyl penicillanate ([^{35}S]methionine label) and purified by interaction with immobilized (E166A) β-lactamase as a function of time. Approximately 20% of the fusion product is immobilized, while little or no binding is seen for the control template.

brary. This starting library contained approximately 1.5×10^{13} unique peptides, based on the efficiency of mRNA peptide fusion formation (data not shown), and thus represented near saturation coverage for the flanking random positions (ten random residues, $20^{10} = 10^{13}$). We confirmed the reduced state of the fixed cysteine by affinity purification on thiopropyl sepharose as in previous work [9] (data not shown).

After coupling the library with sodium 6-bromoacetyl penicillanate, we needed to address two issues: (1) the fraction of the library bearing the drug and (2) whether any other functional groups on the peptide, RNA, or DNA were reactive with bromoacetyl moiety. The drug-modified mRNA display library is indistinguishable from unmodified libraries in standard PAGE experiments because the molecular weight difference between them is too small to be resolved. However, we were able to accurately quantitate the amount of drug-modified library using a hydrolysis-deficient mutant of the RTEM-1 β-lactamase (E166A). This 30 kDa protein forms a stable covalent adduct with penicillin and penicillin derivatives at serine 70 [29].

After library derivatization, we added the mutant β-lactamase (E166A) to the peptide fusion mixture and separated library members that could form a covalent bond with the mutant β-lactamase by electrophoresis (Figure 2A). This analysis revealed that approximately 20% of the mRNA display library members contained a penicillin side chain and also demonstrated that a control mRNA display construct was unreactive with the bromoacetyl derivative of the drug (Figures 2A and 2B). Therefore, our starting library contained $\sim 3 \times 10^{12}$ different peptides bearing the drug as intended. Equally important, the bromoacetyl drug was nonreactive with the functional groups on the template (hydroxyls, phosphates, ring nitrogens, exocyclic amines) and the non-cysteine amino acids (histidine, arginine, asparagine, glutamic and aspartic acid, serine, threonine, glutamine, tyrosine, lysine, and tryptophan) as well as the N-terminal amine in the peptide. Finally, these experiments demonstrate that the penicillin side chain is capable of covalent attachment interaction with the active site of lactamase when covalently attached to a great variety of peptide chains.

Selection for Interaction with PBP2a

The penicillin-tagged fusion library was subjected to iterative cycles of selection for binding immobilized

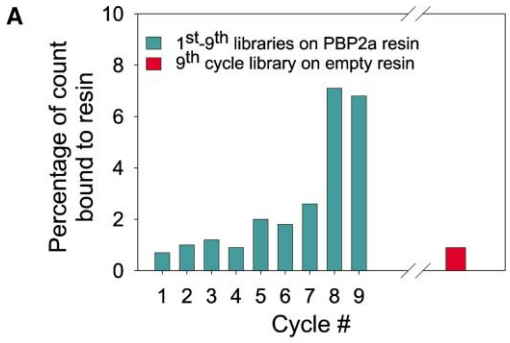

Figure 3. Results of mRNA-Display Selection

(A) Progress of the selection experiment. The PBP2a binding increases in the eighth round and remains constant in the ninth round (green bar; [^{35}S]methionine label]. The ninth-round mRNA-dispay pool shows little or no binding to the empty matrix (red bar).

(B) Sequences of 15 clones from the library after nine cycles of selection. A hyphen is shown for the fixed cysteine in the library and occurs in all the selected sequences. A dot is shown for deletions, and mutations in the constant region are indicated with the one-letter code. Cysteines occurring in the randomized regions are indicated. The sequence used for inhibition testing is indicated in red and appeared twice in the 15 clones.

PBP2a (Figures 1B and 3A). In each round, the library was eluted specifically by liberating the PBP2a from the matrix with DTT (see Experimental Procedures). As the selection progressed, we gradually increased the stringency by decreasing the incubation time of the library on the PBP2a matrix from 1 hr in the first cycle to 10 min in the ninth cycle. Penicillin forms a stable adduct with PBP2a, albeit very slowly under normal conditions. Our selection protocol thus selected for those members that could bind *and* react efficiently. After eight cycles of selection and amplification, the fraction of the library bound to the PBP2a matrix rose significantly above background. An additional ninth cycle resulted in no marked improvement. Under standard conditions where libraries were incubated for 30 min, the fraction of library bound to the PBP2a increased from 0.4% of the first-cycle to 7% of the ninth-cycle library. This interaction was specific for PBP2a, as little binding is seen when the round nine library was tested with solid support alone ($\sim$0.6% binding; Figure 3A).

We cloned and sequenced 15 individuals from the ninth-cycle library (Figure 3B). All of the sequences were found to contain a cysteine at the fixed position. Two of the clones contained two cysteines, indicating that multivalency may increase the chance of a particular peptide to become attached, although this did not appear to be the predominant interaction mode in the library. We found no obvious sequence consensus between these clones, implying many distinct peptides may be able to enhance the binding or reactivity of the β-lactam core.

Inhibition Activity of Selected Sequences

The peptide sequence LRNSNCIRHFF occurred twice in the 15 clones and was chosen for our PBP2a inhibition studies. We chemically synthesized milligram quantities of the peptide-penicillin conjugate (LRNSNC[Pen]-IRHFF; Figure 4), purified the compound by HPLC, and confirmed the identity of the product by mass spectrometry (see Experimental Procedures). We also constructed a version of the peptide lacking the drug as a negative control. To do this, we derivatized the central cysteine moiety with bromoacetic acid to preclude the intermolecular disulfide bond formation and mimic the negative charge on the penicillin carboxylate (LRN SNC[COOH]IRHFF; Figure 4). This blocked peptide product was purified and analyzed as well. We then sought an assay to compare the activity of (1) the peptide-drug conjugate, (2) the blocked peptide, (3) the parent penicillin (6-APA), and (4) cefotaxime, a cephalosporin-based β-lactam. The inhibition of penicillin binding proteins occurs via the following three-step mechanism: (1) association and dissociation of the drug from the enzyme ($K_d = k_{-1}/k_1$), (2) covalent acylation of the active site serine (k_2), and (3) deacylation of the enzyme (k_3) [30].

$$\mathrm{PBP + Pen} \underset{}{\overset{K_d}{\rightleftharpoons}} \mathrm{PBP{\bullet}Pen} \xrightarrow{k_2} \mathrm{PBP\text{-}Pen}$$

$$\xrightarrow[H_2O]{k_3} \mathrm{PBP + Penicilloate}$$

In general, the k_3 is extremely slow ($\sim 10^{-5}\ S^{-1}$) for PBPs. Covalent inhibition of the enzyme thus reflects only a combination of binding ($K_d = k_{-1}/k_1$) and reactivity (k_2).

We measured covalent inhibition (IC_{50}) using a competition-inhibition assay [31]. In this assay (Figure 5A), various concentrations of drug are allowed to react with the protein for a fixed time interval (15 min). Biotin-ampicillin, which can covalently attach to the active site serine, is then added as a competitor, followed by quenching of the reaction. The biotin-ampicillin-PBP2a conjugate can then be detected via chemiluminescent Western blotting. Compounds that block or become covalently attached to the active site of the protein decrease the amount of biotinylated ampicillin that becomes attached to the protein, giving a characteristic inhibition curve and a corresponding IC_{50}. The IC_{50} measurement thus represents a combination of both the equilibrium constant for enzyme substrate complex formation (k_1/k_{-1}) and the formation of covalent drug-enzyme complexes (k_2).

We found that the relative IC_{50} of this peptide conjugate (LRNSNC[Pen]IRHFF) in our assay conditions was about 7 mM, making it a better inhibitor of PBP2a than either unconjugated peptide (LRNSNC[COOH]IRHFF) ($IC_{50} > 35$ mM) or 6-aminopenicillanic acid ($IC_{50} > 500$

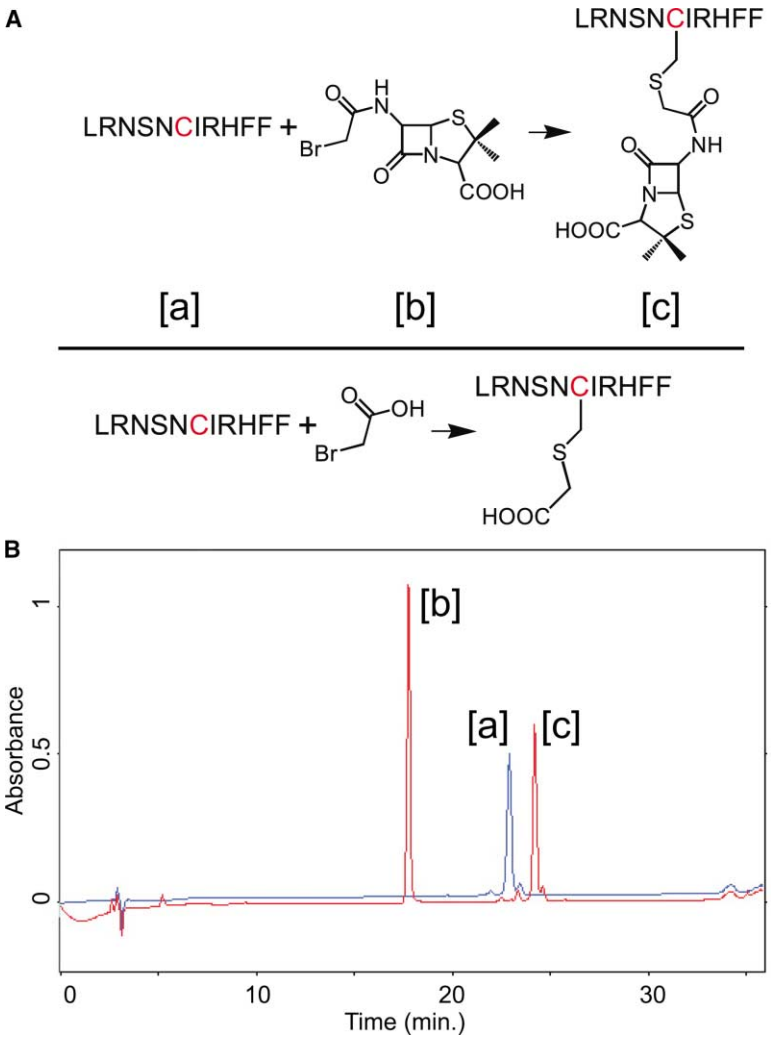

Figure 4. Preparation and HPLC Chromatogram of Peptide-Penicillin Conjugate

(A) Peptide (LRNSNCIRHFF) reacts with sodium 6-bromoacetyl penicillanate or bromoacetic acid to produce peptide-penicillin conjugate (LRNSNC[Pen]IRHFF) and blocked peptide (LRNSNC[COOH]IRHFF), respectively.

(B) The HPLC chromatogram is the superimposed image of traces from two runs. Compound a is the peptide itself, and compound b is the sodium 6-bromoacetyl penicillanate. Product c is the peptide-penicillin conjugate. The HPLC chromatogram indicates that the conversion of peptide to peptide-penicillin conjugate is quantitative and specific, as 100% peptide becomes conjugate without any other side products.

mM) alone (Figure 5B). A control peptide conjugate (EQ KLIC[Pen]SEEDL) that did not appear in the final enriched library showed no such improvement ($IC_{50} > 35$ mM). Our results indicated that substitution of the penicillin core with an appropriate 11-residue oligopeptide greatly enhanced the efficacy of the drug against PBP2a. This enhancement likely occurs via interactions with the peptide and the protein near the active site, as the unmodified peptide also shows detectable activity. While the peptide conjugate (LRNSNC[Pen]IRHFF) is more than 100-fold improved compared to 6-APA, our selected molecule is also comparable to cefotaxime ($IC_{50} =$ 2.9 mM), a cephalosporin compound. Traditionally, the cephalosporin core structure has been the starting point for design of high-affinity inhibitors of PBP2a because these compounds are usually more potent against PBP2a than penicillin systems. Recent structural work on PBP2a indicates that the substituted cephalosporin ring has additional van der Waals contacts with the active site of PBP2a, providing higher affinity than the penicillin ring system [32]. Our results imply that the selected peptides attaching to the 6 position of the penicillin moiety are able to compensate for the absence of these extra van der Waals interactions only seen in cephalosporin systems by contacting the surface area of PBP2a.

The modest IC_{50} (7 mM) that we observe does not necessarily imply that binding of our substrates is correspondingly weak. First, the absolute value of the IC_{50} depends on the conditions chosen for the assay, with longer conjugate-PBP2a incubation times producing correspondingly smaller IC_{50} values. Additionally, the rate constant for covalent attachment of penams to PBP2a (k_2) is quite slow, ranging from $k_2 = 0.22\ s^{-1}$ for benzyl penicillin to $k_2 = 0.0083\ s^{-1}$ for methicillin [30]. For comparison, the rate constant for formation of the acyl intermediate between penams and β-lactamase is $\sim$2000 s^{-1}, 10^4- to 10^6-fold faster than PBP2a [33]. Covalent attachment of our compounds depends on the ratio of k_{-1}/k_2, as this value reflects how bound drug will partition between dissociation and product formation. Values for k_1, the formation rate constant, are typically in excess of $10^8\ M^{-1}s^{-1}$ for penams interacting with β-lactamase [33]. If we presume that $k_{-1} \cong k_2$ in order to give significant product formation, the predicted value of K_d would be nanomolar or below. If we conservatively estimate that k_1 is $10^6\ M^{-1}s^{-1}$, typical for macromolecular association reactions, we predict values of K_d that are micromolar or below. Overall, the biochemical analysis indicates that the appended peptide facilitates the desired function of the drug by at least 100-fold. This ob-

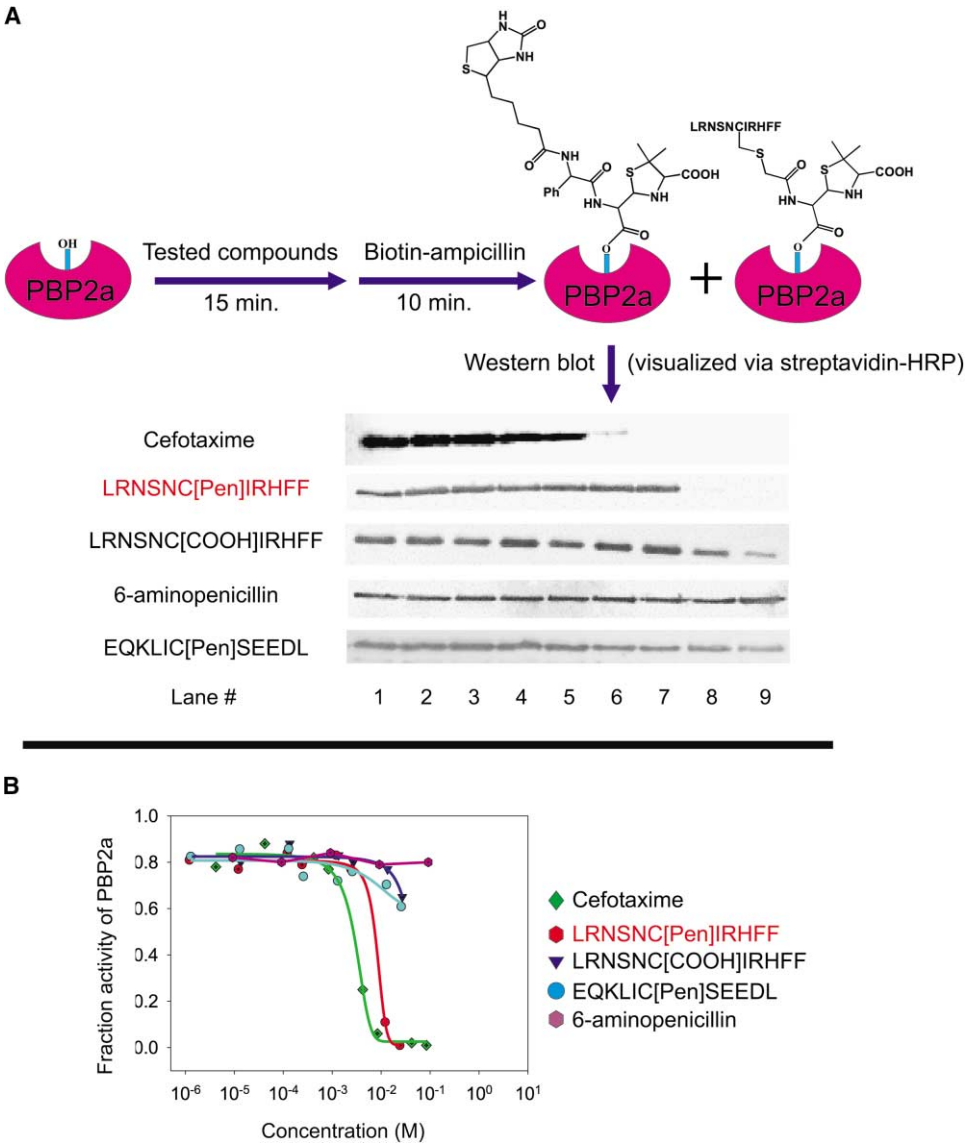

Figure 5. Functional Analysis of Penicillin and Penicillin-Drug Conjugates

(A) Scheme of competition assay with biotin-ampicillin and functionality of penicillin-peptide conjugates. The drug concentrations in the inhibition assays were as follows: lane 1, 0 μg/ml; lane 2, 2 μg/ml; lane 3, 20 μg/ml; lane 4, 200 μg/ml; lane 5, 400 μg/ml; lane 6, 2 mg/ml; lane 7, 4 mg/ml; lane 8, 20 mg/ml; and lane 9, 40 mg/ml.

(B) Inhibition curves for the molecules tested.

servation is therefore consistent with our peptides adding approximately 3 kcal to the stabilization (k_1/k_{-1}), the reactivity (k_2), or some combination of the two to the parent penam drug.

Significance

Our overall goal in this work was to extend the chemical diversity possible in mRNA-display libraries through creation of functional drug-peptide conjugates. To that end, we have demonstrated that a penicillin side chain may be appended to a mRNA-display library in a chemically orthogonal fashion with reasonable synthetic efficiency. After the nine-round selection, all of the cloned sequences were in-frame and contain a cysteine residue at the fixed position. These observations are consistent with the idea that the peptide-drug conjugate was formed and selected for interaction with the PBP2a, targeting our peptide library to the active site of the protein. Chemical synthesis of the peptide-drug conjugate confirms that this compound is in fact active against PBP2a, whereas both the drug and the peptide only show little or no activity under concentrations that can be examined experimentally.

Perhaps the most compelling aspect of these experiments is their generality. There are numerous examples where drugs target specific sites in proteins [34,

35]. The ability to generate peptide-drug conjugates enables the use of small molecules to direct display libraries to a particular face or site in a protein target of interest. Additionally, the covalent derivatization strategy we have used here enables library construction using compounds that would not be possible via in vitro nonsense suppression [17]. For example, compounds that strongly inhibit translation, such as kinase inhibitors or GTP analogs, or side chains that might be too large to fit in the exit tunnel of the ribosome (e.g., a heme) may not be efficiently inserted via nonsense suppression. We therefore anticipate that our approach should be broadly applicable for the development of novel tools to control biochemical processes.

Experimental Procedures

Preparation of Sodium 6-Bromoacetyl Penicillanate

Four hundred thirty-two milligrams of 6-aminopenicillanic acid (Sigma; 6-APA, 2 mM) and 500 mg sodium bicarbonate (Sigma; 4.5 mM) were mixed in 4 ml water and 2 ml acetone with stirring for 10 min at 0°C. Four hundered four milligrams of bromoacetyl bromide (Aldrich; 2 mM, 175 μl) was dissolved in 2 ml of acetone and added to the stirring solution. The reaction flask was wrapped with aluminum foil and kept on ice for 10 min more. The solution was removed from the ice bath and allowed to come to room temperature. The reaction was stirred for 1 hr, and 5 ml of water was added to dissolve any residual white salt in the flask. The reaction was extracted twice with ether (4 ml) and then covered with 5 ml ethyl acetate. The reaction mixture was acidified with 40% phosphoric acid with stirring at 0°C. The ethyl acetate layer was removed, extracted (3×) with 5 ml distilled water, and dried over anhydrous magnesium sulfate. The dried reaction mixture was combined with 300 μl n-butanol containing sodium 2-ethylhexanoate and stirred for 30 min. The precipitate was collected by filtration, washed with several portions of ethyl acetate, and air dried (395 mg). The ESI-MS gives two equal peaks at 335.0 and 337.0. (The expected mass peaks $[M - H]^-$ are 334.97 and 336.97.)

Preparation of mRNA-Peptide β-Lactam Fusion

A synthetic DNA library template sd7 (ACTATTTACAACCACCATG NNSNNSNNSNNSNNSTGCNNSNNSNNSNNSNNSGGCGGCGACT AAGGACGACGATGACAAGGCGGCGGCGGC) was purified by preparative polyacrylamide gel electrophoresis and amplified by polymerase chain reaction (PCR) with the primers sd2 (GGATTCTAATAC GACTCACTATAGGGACAATTACTATTTACAACCACCATG) and sd3 (GCCGCCGCCGCCCTTGTCATCGTCGTCCTTGTAGTC). The resulting double-stranded DNA template was used to prepare the mRNA display fusion library as previously described [28]. After purification with oligo-dT cellulose (New England Biolabs), the fusions were used as templates for reverse transcription at conditions recommended by the manufacturer (Superscript II RNase H^- reverse transcriptase, BRL, Life Technologies). The product was purified by phenol extraction and ethanol precipitation and dissolved in 300 μl reaction buffer (100 mM sodium borate, pH 8.3). Then, 8 μl tris(2-carboxyethyl) phosphine (TCEP; 20 mM, pH 8.0) was added and incubated at room temperature for 30 min. Sodium bromoacetyl penicillanate (1.1 mg; 10 mM final solution) was dissolved in this solution and the mixture was shaken for 1 hr in the dark. To quench the reaction, 30 μl 2M DTT was added, and the reaction was stirred for 30 min. The fusion conjugate (penicillin-modified cDNA-mRNA-peptide fusion) was then ethanol precipitated and redissolved for use in the selection experiment.

Selection against Immobilized PBP2a

The truncated PBP2a [23] gene was inserted into PTXB3 plasmid (New England Biolabs) between NCoI and SapI sites, then transformed into *E. coli* BL21(DE3) strain. Bacteria were grown in LB medium at 37°C until A_{600} reached 0.6 and induced with 1 mM IPTG. After growing 3 hr more at 37°C, the bacteria were harvested for purification of PBP2a protein using chitin beads at conditions recommended by the manufacturer (New England Biolabs). The PBP2a protein was cleaved from beads with 50 mM DTT in column buffer (20 mM HEPES, pH 8.0; 500 mM NaCl; 1 mM EDTA) and dialyzed against phosphate buffer (50 mM, pH 8.0) overnight.

The pure PBP2a was reacted with biotin-SS-(sulfo)-NHS (Pierce Chemical; 10 mM in 50 mM phosphate buffer, pH 8.0) and dialyzed against phosphate buffer (50 mM, pH 8.0). The resulting biotinlyated PBP2a was incubated with streptavidin agarose gel (Pierce Chemical) and shaken for 30 min. The supernatant was removed, and the resulting PBP2a agarose was washed with phosphate buffer. The fusion conjugate was mixed with immobilized PBP2a agarose in 1 ml of incubation buffer (50 mM phosphate, pH 8.0; 100 mM NaCl; 2 mM biotin) and incubated for variable time (cycle 1–3, 1 hr; cycle 4–7, 30 min; cycle 8 and 9, 10 min). The agarose then was washed six times with 500 μl washing buffer (50 mM phosphate, pH 8.0; 100 mM NaCl; 0.1% Triton X-100). Five hundred microliters cleavage buffer (50 mM phosphate, pH 8.0; 100 mM NaCl; 100 mM DTT) was incubated with washed agarose for 1 hr. The supernatant was collected, desalted, concentrated with microcon YM-30 (Milipore), and used for PCR amplification directly with sd2 and sd3 as primers.

Relative IC_{50} Assay of Peptide Penicillanate Conjugates

The selected peptides were synthesized in an ABI peptide synthesizer with Fmoc chemistry, deprotected with TFA, and purified by HPLC. The lyophilized peptides were reacted with sodium bromoacetyl penicillanate in sodium borate buffer (100 mM, pH 8.3). The conjugates of peptide with β-lactam were purified again by HPLC and lyophilized. Various concentrations of conjugates were prepared by dissolving in reaction buffer (50 mM phosphate, pH 8.0; 100 mM NaCl) and incubating with 2 μg pure PBP2a, respectively, at 37°C for 15 min. Biotin-ampicillin (20 μg/ml) was added immediately and incubated at 37°C for 10 min. The reaction was stopped by heating the reaction mixture at 94°C for 5 min. The samples were loaded into 12% SDS-PAGE for separation, then transferred to a nitrocellulose membrane by blotting with sodium bicarbonate buffer (20% methanol; 3 mM Na_2CO_3; 10 mM $NaHCO_3$) at 400 mA for 3 hr. The membranes was blocked with blocking solution (20 mM Tris, pH 8.0; 150 mM NaCl; 0.05% Tween-20; 5% milk) for 1 hr, then incubated with 10 ml TBS buffer (20 mM Tris, pH 8.0; 150 mM NaCl) containing 1 μg/ml streptavidin-HRP protein (Pierce Chemical) at room temperature for 1 hr. The membrane was washed with 50 ml TBS buffer six times at 5 min intervals and covered with femto-Western blotting reagent (Pierce Chemical). The membrane was then detected under the digital camera in the dark to collect chemiluminescent emissions for 10–15 min.

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