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Use of combinatorial library screening to identify inhibitors of a bacterial two-component signal transduction kinase

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Summary

Bacterial resistance to antibiotics is emerging as a major concern to the medical community. The appearance of several antibiotic-resistant strains, including multidrug-resistant *Staphylococcus aureus*, raises the prospect that infections by these bacteria could soon become untreatable with currently available antibiotics. In order to address this problem, increased emphasis is being placed on the discovery of novel classes of antibacterial agents that inhibit novel molecular targets using sources of compounds not yet exploited for antibiotic drug discovery. Novel classes of compounds can now be rapidly investigated using combinatorial chemistry approaches. This report describes the identification of novel antibacterial compounds from a combinatorial library of N-acetylated, C-amidated D-amino acid hexapeptides. This library of compounds was screened for inhibitors of CheA, a member of the bacterial two-component signal transduction kinase family. Several peptides with apparent IC_{50} values in the low micromolar range were identified. In addition to inhibiting CheA, these peptides inhibited mammalian protein kinase C (from rat brain) with comparable potency. Finally, these peptides were also found to have significant antibacterial properties, although the true mechanism by which they exhibited inhibition of bacterial growth remains uncertain.

Abbreviations: Single letter-codes are used for amino acid residues.

Introduction

In recent years, the process of screening to identify new compounds with desirable pharmaceutical properties has undergone a revolution. Recent advances in molecular and cellular biology have enabled the identification, cloning, and expression of numerous unexploited targets for the development of new screening assays. Improvements in assay automation and computerized data processing have also allowed an enormous increase in the throughput of biological assays. In addition, the advent of combinatorial chemistry has further led to a substantial increase in the effective throughput of an assay. Development of combinatorial chemistry has allowed the generation of large numbers of individual compounds either in arrays of single compounds or in pools of mixtures, which have substantially increased the number and diversity of compounds available for screening [1,2]. In particular, mixture-based combinatorial libraries allow the simultaneous screening of thousands of individual compounds of a given chemical class. The individual active compounds are subsequently identified following a systematic deconvolution process [3–7]. Overall, these advances have the potential to impact all areas of drug discovery.

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In the area of antibacterial therapeutics, resistance to currently available drugs is progressively limiting their utility in treating bacterial infections. This problem can be addressed by discovering novel drugs that inhibit novel targets, thereby bypassing the existing mechanisms of resistance. Advances in molecular microbiology and genomics have led to the identification of numerous bacterial genes coding for novel proteins that could potentially serve as targets for novel antibacterial compounds. Regulatory proteins such as the two-component histidine kinases, involved in bacterial signal transduction, have recently gained considerable attention as one such class of potential targets [8–11]. However, little is known about the properties of bacterial two-component histidine kinases to enable the design of potent inhibitors of these kinases which are structurally and mechanistically different from the extensively studied eukaryotic signal transduction kinases.

In an effort to identify generic inhibitors of the bacterial two-component signal transduction kinase family, a screening assay in 96-well format was developed using CheA, the kinase involved in bacterial chemotaxis in *Escherichia coli*, as a prototype enzyme [12]. To circumvent the drawbacks inherent in the use of L-amino acids (low in vivo stability), mixture-based combinatorial hexapeptide libraries of D-amino acids were selected to identify novel inhibitors of CheA. While approximately 50 million individual peptides were present in these libraries, the pooling of these peptides into mixtures resulted in the screening of only hundreds of separate samples. Mixture-based libraries have been proven successful in a large number of examples described in the literature, such as the successful screening of D-amino acid hexapeptide libraries for the identification of novel high affinity ligands of the μ opioid receptor [4]. In the area of antibacterial drug discovery, the identification of antimicrobial and/or antifungal peptides, peptidomimetics, and heterocyclic compounds from mixture-based combinatorial libraries have also been reported [5,13–15].

Two different deconvolution strategies described in the literature were used in this study: (1) Iterative deconvolution: this process involves the successive identification of each residue (building block) of the active sequence(s). At each step, the number of defined residues increases as the number of compounds per mixture decreases [5,6]. (2) Positional scanning: this process involves the screening of separate sublibraries in which the position of the defined amino acid(s) (building block(s)) differs from one sublibrary to another. The number of sublibraries depends on the length of the sequence and/or number of positions defined. In the case of the hexapeptide library used in this work, there were six sublibraries, each with a single position (1 through 6) defined. Each sublibrary contained all the peptides of the library pooled in 20 separate mixtures, each with a different D-amino acid residue in the defined position. The data derived from screening of each sublibrary yield information on key residue(s) at every position. As a result, the sequence(s) of the active peptides can be identified in a single screening assay [7]. Unlike the iterative approach, the positional scanning approach has the potential to identify the sequence (structure) of active compounds in a single step. In this report, we describe the use of these two deconvolution strategies to identify several peptide inhibitors of CheA, while demonstrating the advantages and disadvantages of each strategy.

Methods

Materials

The microtiter plates with nitrocellulose filters were purchased from Millipore Corporation (Bedford, MA). Resins for protein purification were obtained from Pharmacia (Piscataway, NJ). [γ-^{33}P]ATP was purchased from Amersham (Arlington Heights, IL). Purified protein kinase C (from rat brain) and its substrate histone H1 (from calf thymus) were obtained from Boehringer Mannheim GmbH (Germany). Bacterial cells were grown in sterile, 96-well, round-bottom, polystyrene microplates (Corning Glass Works, Corning, NY). Sterile saline (0.9% v/v) was used to dilute the inoculum. All other reagents used in this study were of the highest quality and purity available. A Biomek 2000 robotic system (Beckman, Palo Alto, CA) was used for liquid handling and serial dilution purposes.

Bacterial signal transduction kinase (CheA) inhibition

The CheA assay was performed in a 96-well format using Millipore nitrocellulose filter plates following a method described previously [12]. Partially purified preparations of CheA from *Escherichia coli* strain RP437/pDV4 (expressing the *cheA* gene from the *trc* promoter) was used for this work. The CheA kinase

reaction was initiated in a volume of 75 μl per well (containing 20 μM of CheA) by the addition of [γ-^{33}P]ATP. Following a 20-min incubation at room temperature, the reaction was terminated by the addition of 25 μl EDTA (25 mM final concentration) [16]. Next, 150 μl of wash buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl) was added to each well. The filter plate was then subjected to filtration under vacuum to remove the reaction mixtures from the wells. Each well was then washed with 2 ml of wash buffer, followed by 400 μl of H_2O to remove salts. Radioactivity associated with the dried wells was quantified by scintillation counting using a Packard TopCount MicroPlate Scintillation counter (Packard Instrument Co., Meriden, CT).

Inhibition of CheA activity by the test compounds was measured along with control reactions on each 96-well plate using different concentrations of ADP as a control inhibitor at 1–200 μM concentration range exhibiting CheA inhibition at 0–100% range [12]. Each test compound or mixture was tested at three to five different concentrations to produce a dose–response curve. Inhibition by active peptides pursued for further deconvolutions was confirmed by re-testing. IC_{50} values of certain peptides were determined by measuring CheA inhibition at 1–16 μM peptide concentration range, followed by non-linear regression analysis using SigmaPlot software (from SPSS Inc., Chicago, IL).

Antibacterial activity

Determination of Minimum Inhibitory Concentrations (MIC) of test compounds was performed in Brain Heart Infusion (BHI) broth (Beckton-Dickinson, Cockeysville, MD). Bacterial cultures were prepared from a petri dish (with Brain/Heart Infusion [BHI] agar) incubated overnight at 37 °C. Colonies from the petri dish were resuspended in sterile physiological saline to an optical density (measured at 600 nm) of 0.1–0.12. This bacterial suspension was further diluted 1:50 into BHI media to yield a suspension of 2×10^6 colony forming units (cfu) per ml and 100 μl of this suspension was added to the wells of a 96-well plate microtiter plate. A 100-μl aliquot of the test compound, at a concentration twice that of the desired level, was then added to the wells. The microtiter plate was then incubated for 18–24 h at 37 °C, and the MIC was recorded as the lowest concentration of test compound inhibiting visible growth of bacteria [17].

The D*-amino acid hexapeptide library*

A combinatorial library of N-acetylated, C-amidated hexapeptides of D-amino acids was generated in an iterative format as described elsewhere [3]. The iterative format was composed of $\sim 52.13 \times 10^6$ peptides in total, divided into 400 mixtures, each containing defined amino acid residues at the first two N-terminal positions. The defined positions represented all possible permutations (20^2) of 20 D-amino acids (glycine and D-isomers of all natural amino acids). The remaining four positions comprised approximately equimolar mixtures of 19 D-amino acids (glycine and D-isomers of all natural amino acids except cysteine). Following the screening of these 400 mixtures, the data were used to identify the residues at these two positions that defined the mixture(s) exhibiting the most potent inhibition of CheA. Subsequent steps involved the synthesis and screening of 20 mixtures at a time, each containing an additional defined amino acid, until the individual peptides were identified. In the initial screening of 400 mixtures, each contained ~130 321 (= 19^4) individual peptides. In each successive round of screening, 6859 (= 19^3), 361 (= 19^2), 19 (= 19^1), and 1 peptides were present per mixture.

A similar combinatorial library was designed in a positional scanning format, each mixture with a single position defined [4,7]. The defined positions represented 20 D-amino acids (glycine and D-isomers of all natural amino acids) while the remaining five positions comprised approximately equimolar mixtures of 19 D-amino acids (glycine and D-isomers of all natural amino acids except cysteine). This library was composed of $\sim 49.52 \times 10^6$ peptides in six sublibraries (with defined residues in positions 1 through 6), each consisting of 20 mixtures, each of which contained $\sim 2.47 \times 10^6$ (= 19^5) peptides with a defined amino acid in a given position. This format allowed the simultaneous identification of the key residues at each position. As a result, the screening of the 120 (= 6×20) mixtures led to sufficient information needed to identify the individual inhibitory compounds in a single step. Peptides representing all possible combinations of selected key residues were then synthesized and screened to determine their activity.

The libraries were prepared by standard Merrifield solid phase peptide synthesis methodologies [18] using the simultaneous multiple peptide synthesis technology [SMPS] [19]. A predetermined chemical ratio of amino acids was used to prepare the resin-bound mixtures, while the defined positions were added

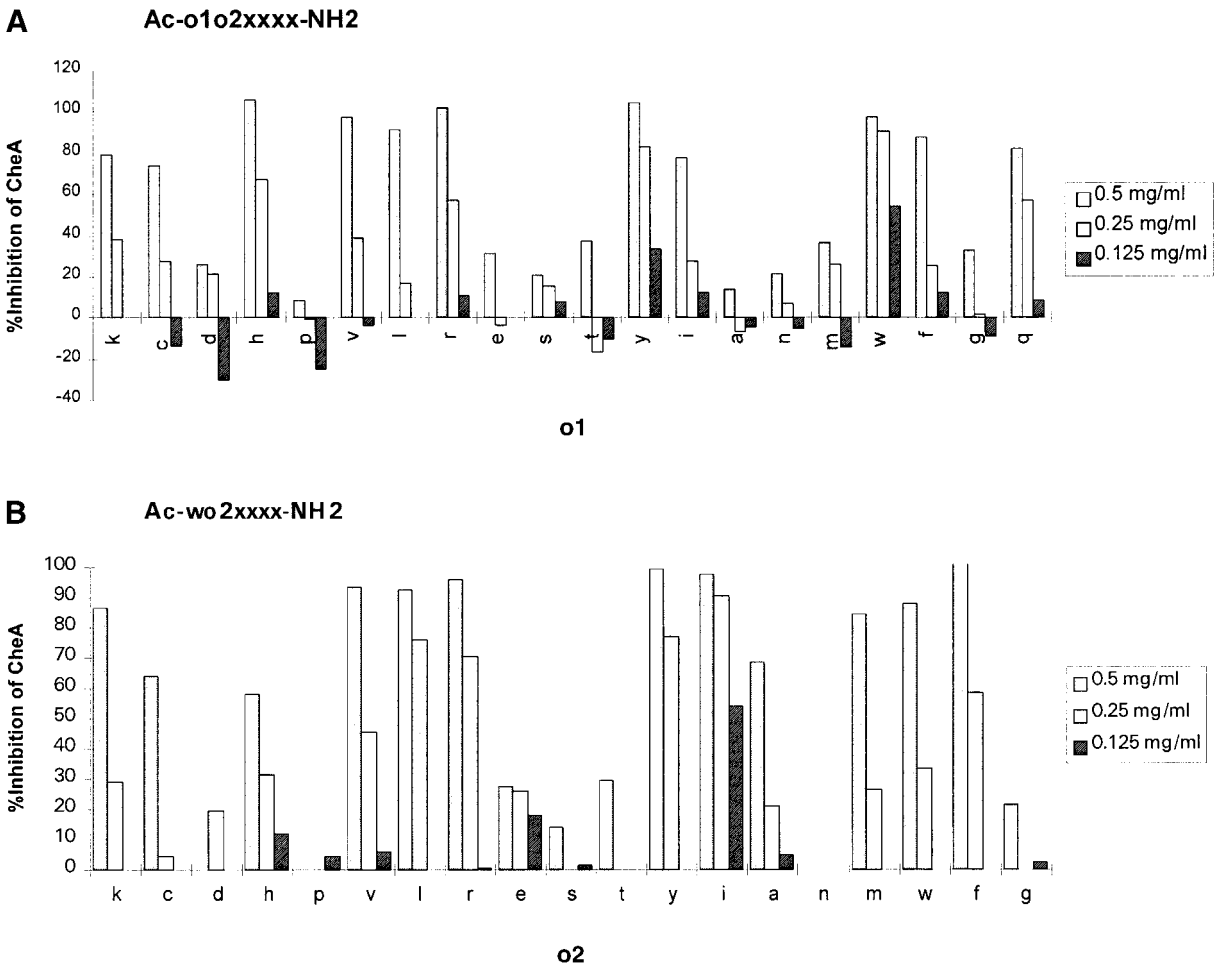

Figure 1. Screening of the iterative hexapeptide combinatorial library for inhibition of CheA activity. Each mixture is defined at the first two positions (o1 and o2). The remaining four positions represent a mixture of 19 D-amino acids (position x, described under Methods). (A) The highest inhibitory activity found for each of the 20 sets of mixtures defined by the amino acid at position 1 (o1) is shown. (B) The inhibitory effect of each of the 20 mixtures defined with D-tryptophan (w) at position 1 (o1) and 20 D-amino acid residues in position 2 (o2) is shown.

individually using SMPS [20]. Final cleavage and deprotection steps were carried out with liquid hydrogen fluoride (HF) using a 'low-high' HF cleavage protocol [21,22]. The mass distribution of a number of randomly selected mixtures was confirmed using a Kratos MALDI-TOF laser-desorption time-of-flight spectrometer. Separate mixtures of 19 peptides, each generated by incorporating a mixture of 19 D-amino acids at a given position of a control sequence, were synthesized. A set of individual peptides was also synthesized along with the libraries to control the incorporation of the 19 D-amino acids at the mixture positions, as well as the coupling steps and cleavage of the libraries, respectively. The identity and purity of these individual peptides were analyzed by mass spectral analysis interfaced with a liquid chromatography system (Finnigan LCQ). The quality and reproducibility of the synthesis of our mixture-based peptide libraries were earlier validated using the corresponding L-amino acid hexapeptide libraries. Thus, known ligands to the opioid receptor [23,24] and antigenic determinants [3,7] that were theoretically present in the libraries were readily identified using both the iterative and positional scanning deconvolution processes. Such case studies could not be performed with the D-amino acid hexapeptide libraries since no known D-amino acid hexapeptide with biological activity in assays, currently performed in our laboratory, has been reported.

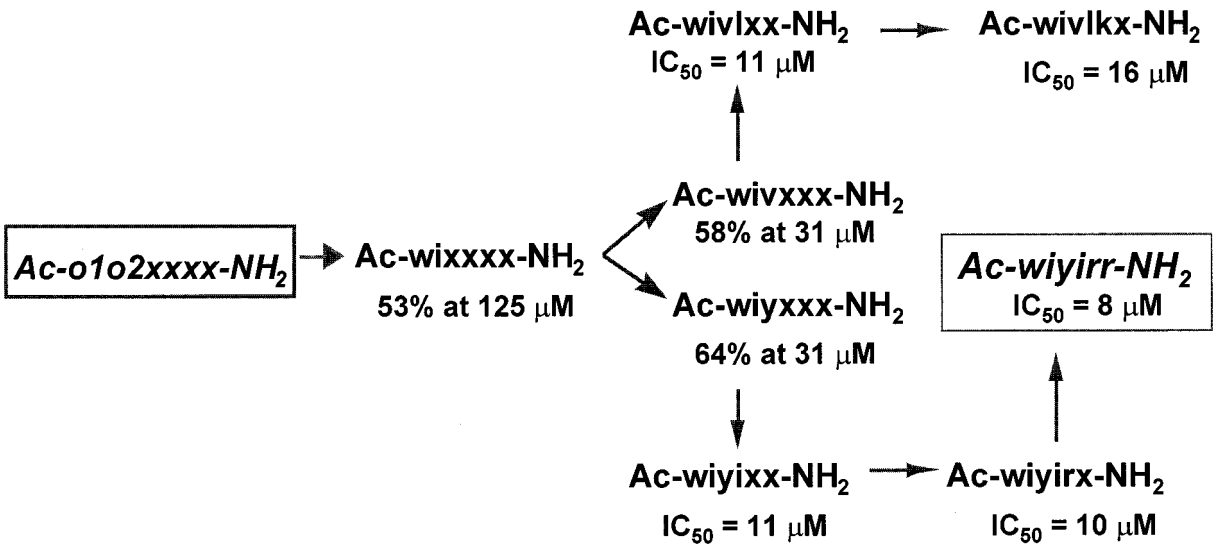

Figure 2. Summary of the deconvolution pathway followed to obtain the final inhibitor. As described in the text, 'x' represents positions with an approximately equimolar mixture of residues. Inhibition potency of each peptide is shown either as % inhibition of CheA activity at a given concentration (for Ac-wixxxx-HN_2, Ac-wiyxxx-NH_2, and Ac-wivxxx-NH_2), or as IC_{50} values (for Ac-wiyixx-NH_2, Ac-wivlxx-NH_2, Ac-wiyirx-NH_2, Ac-wivklx-NH_2, and Ac-wiyirr-NH_2).

Results

To identify the key residues required at the first two N-terminal positions to inhibit the CheA kinase, the 400 mixtures of the iterative library were screened. As shown in Figure 1A, peptide mixtures defined with aromatic or positively charged D-amino acids at position 1 (o1) showed the highest potencies of CheA inhibition. On the other hand, peptide mixtures defined with residues such as glycine (g), D-alanine (a), D-serine (s), D-threonine (t), and D-cysteine (c) in position 1 showed relatively low levels of CheA inhibition. The highest level of inhibitory potency was obtained when the first position was defined with D-tryptophan (w). When D-tryptophan (w) was defined at position 1 (Ac-woxxxx-NH_2), the most active mixture was defined with D-isoleucine (i) at position 2 (Ac-wixxxx-NH_2) as shown in Figure 1B. Interestingly, several other mixtures with D-isoleucine (i) in position 2 (Ac-oixxxx-NH_2) showed higher levels of CheA inhibition relative to corresponding mixtures with other residues in position 2 (data not shown). Subsequent rounds of deconvolution were continued with the mixture Ac-wixxxx-NH_2.

To define the third position, 20 new mixtures were synthesized using the formula Ac-wioxxx-NH_2 where 'o' represents a defined residue. As shown in Figure 2, two mixtures, Ac-wivxxx-NH_2 and Ac-wiyxxx-NH_2, were found to have similar activity against CheA and were chosen for further deconvolution. Iterative screening to identify the best residue in the fourth position was continued using two sets of mixtures (20/set) defined as Ac-wivoxx-NH_2 and Ac-wiyoxx-NH_2. Ac-wivlxx-NH_2 and Ac-wiyixx-NH_2 were found to be most active as CheA inhibitors. The next round of iterative screening identified two mixtures, Ac-wivlkx-NH_2 and Ac-wiyirx-NH_2, with the fifth position defined for the highest levels of inhibitory activity against CheA (Figure 2). Ac-wivlkx-NH_2 was not chosen for further deconvolution because of the observed decrease in potency as a result of deconvolution. In the final step of iterative deconvolution, a set of 20 individual peptides, defined as Ac-wiyiro-NH_2, was screened to identify the best residue at position 6. At this position, positively charged amino acid residues, D-arginine (r), D-lysine (k), D-histidine (h), and D-threonine (t) were found to yield the most potent peptides with apparent IC_{50} values of 8, 14, 16, and 13 μM, respectively (Figure 3). Therefore, Ac-wiyirr-NH_2 was the most potent inhibitor of CheA activity from this series.

In addition to iterative deconvolution, the hexapeptide positional scanning library was also screened for inhibition of CheA activity. The 120 peptide mixtures were screened in the CheA kinase assay and the activities of the three most potent mixtures from each of the sublibraries are shown in Table 1. Based on these results, an initial set of 16 individual pep-

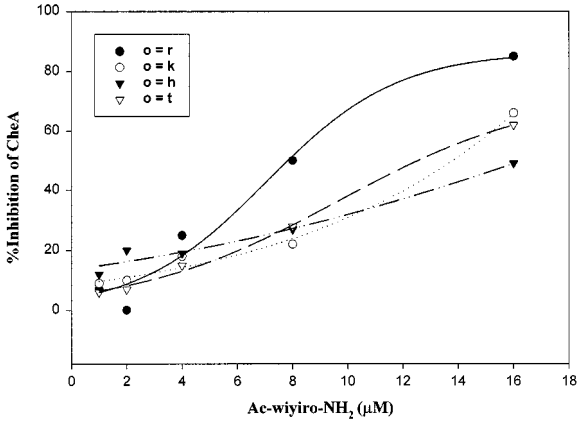

Figure 3. Inhibition of CheA activity at the final step of iterative deconvolution. Dose–response curves for peptides with D-arginine (r), D-lysine (k), D-histidine (h), and D-threonine (t) at position 6 (o), are shown. IC_{50} values were generated by nonlinear regression analyses of the data.

tides representing combinations of key residues was generated: D-isoleucine (i) at positions 1 and 2, D-isoleucine (i) and D-valine (v) at position 3, D-tyrosine (y) and D-isoleucine (i) at position 4, D-leucine (l) and D-isoleucine (i) at position 5, and D-leucine (l) and D-phenylalanine (f) at position 6. However, none of these peptides showed a >50% inhibition of CheA activity at a 100 μM concentration (data not shown). This is apparently attributable to a lack of connectivity between the residues defining the most active mixtures (i.e., the observed activities are not due to a single family of peptides).

To identify those peptides responsible for the activity seen in the positional scanning library, the results from the iterative deconvolution for the N-terminal position were combined with those from the screening of the positional scanning library. Thus, a set of 51 individual peptides was synthesized with a D-tryptophan (w) at position 1 (key residue identified from the iterative library) and a combination of key residues (and their analogs) identified from the screening of the positional scanning at the remaining five positions (Table 1). D-isoleucine (i) and D-phenylalanine (f) at position 2, D-isoleucine (i), D-valine (v), D-tyrosine (y), and D-tryptophan (w) at position 3, D-isoleucine (i), D-phenylalanine (f), and D-tyrosine (y) at position 4, D-isoleucine (i) and D-leucine (l) at position 5, and D-leucine (l), D-tyrosine (y), and D-arginine (r) at position 6 were used to synthesize these peptides. As shown in Table 2, several of these peptides exhibited potent inhibition of CheA activity at low μM concentrations. Peptides with D-leucine (l) and D-arginine (r) at positions 5 and 6, respectively, showed the highest levels of CheA inhibition (48–75% at 8 μM) among all the peptides tested in this series. Inhibition potency of the remaining peptides with other residues at these positions was lower ($\leq$33% at 8 μM). This approach produced Ac-wfvilr-NH_2 as the most potent inhibitor of CheA activity.

Finally, the minimum inhibitory concentrations (MICs) against various bacterial strains were evaluated for two of the most active peptides, Ac-wiyirr-NH_2 and Ac-wfvilr-NH_2, identified by the two approaches described above. As shown in Table 3, the

Table 1. Results of positional scanning[a]

Hexapeptide library	(%) CheA inhibition at 500 μM	250 μM	125 μM
Position 1			
i	45	21	7
l	32	14	8
y	31	12	–4
Position 2			
i	61	37	ND[b]
f	60	32	12
v	41	34	–8
Position 3			
i	73	31	30
f	54	34	ND
y	48	16	–2
v	47	39	22
Position 4			
y	81	37	17
i	74	32	–15
v	62	29	–8
Position 5			
i	58	41	24
l	51	28	12
v	29	18	5
Position 6			
l	43	19	5
y	39	9	8
f	31	17	11
r	31	15	2

[a] Inhibition potencies of the most active mixtures from each of the sublibraries are shown.
[b] ND: Not determined.

Table 2. Results of combining iterative and positional scanning-based deconvolution

Peptide sequence[a]	(%) CheA inhibition at 8 μM
wiyilr	75
wfvilr	73
wiyflr	68
wiwflr	68
wfwilr	66
wivflr	65
wfyflr	65
wfvflr	63
wfwflr	60
wfyilr	52
wiwilr	49
wivilr	48
wfyfll	33
wiyfll	23
wfvily	23
wfyily	23
wfwfly	22
wiyily	21
wfwily	20

[a] All peptides were N-acetylated and C-amidated. Out of 51 peptides tested in this step, only those exhibiting $\geq$20% CheA inhibition are shown.

MICs of Ac-wiyirr-NH_2 against all the *S. aureus* strains were in the 16–64 μM range. Its MICs against *Escherichia coli* strains ES142, DC0, and DC2 (hyperpermeable mutant) were 32, 64, and 16 μM, respectively. *Moraxella catarrhalis* was inhibited at 8 μM and *Klebsiella pneumoniae* strain KL21 at 64 μM. Ac-wfvilr-NH_2 showed more potent overall antibacterial activity than Ac-wiyirr-NH_2, with MICs against several *S. aureus* strains in the 8–16 μM range. Its MICs against *Enterococcus faecium*, a hyperpermeable mutant of *Escherichia coli* (strain DC2), and *Moraxella catarrhalis* were 8–16, 8, and 4 μM, respectively. In addition, this compound failed to show red blood cell lysis activity at 125 μM and is therefore unlikely to be a nonspecific membrane-destabilizing agent. When tested against the mammalian protein kinase C (PKC) from rat brain, we found that the compound was almost as active as it is against CheA (data not shown). Like Ac-wfvilr-NH_2, Ac-wiyirr-NH_2 also showed inhibition of mammalian protein kinase C (PKC) with apparent IC_{50} in the 5–10 μM range and did not cause lysis of red blood cells (data not shown).

Discussion

This paper describes the identification of D-amino acid peptide inhibitors of a bacterial two-component histidine kinase using the combinatorial library screening approach. This represents a novel class of inhibitors for this class of enzymes that are potential targets for novel antibacterial compounds. A number of individual peptides were identified that are not only active against the enzymatic target of the screen but also inhibit growth of several bacterial pathogens. However, the precise mechanism by which these peptides inhibit bacterial growth has not been defined. It is possible that, because of the prevalence of hydrophobic residues in their structure and their conformational flexibility, these peptides are interacting with multiple protein targets or with bacterial membranes to cause an overall inhibition of cell growth. However, the possibility of nonspecific interaction with membranes is not consistent with the observation that these compounds did not lyse red blood cells at concentrations well above their inhibitory concentration (data not shown). The relatively weak antibacterial activity of these peptides against gram-negative pathogens could be due to the inability of these peptides to penetrate two lipid bilayer membranes as opposed to one in the case of gram-positive pathogens.

In screening the hexapeptide combinatorial library for CheA inhibitors, the positional scanning approach revealed its limitations. First, the key residues identified from all the sublibraries were hydrophobic. This may indicate that hydrophobic residues were responsible for inhibition irrespective of their positions within the peptide sequence. In this case, disparate individual peptides could be responsible for the total inhibitory activity observed during the screening of mixtures from the positional scanning library. Thus, while D-isoleucine (i) was found to be a key residue at position 1 based on the position scanning data (Table 1), none of the individual peptides synthesized with this residue at position 1 was a potent inhibitor. In addition, use of relatively high concentrations (up to 500 μM) of peptide mixtures could have caused nonspecific inhibition via hydrophobic interactions with the protein target. It should also be noted that the iterative screening approach has its own limitations. Depending on the position at which deconvolution is initiated, it may be possible to reach different optimum sequences reflecting the 'local minima' favored by the first residue to be defined. The compounds identified by this method could therefore reflect the highest potency based on

Table 3. Antibacterial activity of deconvoluted peptides

Bacterial species	Resistance/ sensitivity[a]	Strain	MIC (μM)	
			Ac-wiyirr-NH_2	Ac-wfvilr-NH_2
Staphylococcus aureus	MSCS	MI246	16	8
Staphylococcus aureus	MSCS	MI273	64	16
Staphylococcus aureus	MSCR	MI345	32	16
Staphylococcus aureus	MRCS	MI300	64	16
Staphylococcus aureus	MRCR	MI339	64	16
Staphylococcus saprophyticus		MI276	16	4
Staphylococcus epidermidis	MSCR	SE48	16	8
Enterococcus faecium	VS	EF1	16	8
Enterococcus faecium	VR	EF12	16	16
Enterococcus faecalis		STD44	32	8
Streptococcus pneumoniae		STP1	8	4
Streptococcus pyogenes		STA2	8	2
Streptococcus viridans		STV1	16	ND[b]
Streptococcus pneumoniae		STP6301	16	4
Escherichia coli		ES142	32	>64
Escherichia coli		DC0	64	>64
Escherichia coli		DC2	16	8
Moraxella catarrhalis		BC2	8	4
Klebsiella pneumoniae		KL21	64	64
Klebsiella pneumoniae	CR	KL328	>64	>64
Proteus mirabilis		PR91	>64	>64
Pseudomonas aeruginosa		PS96	>64	>64

[a] MSCS: Methicillin-sensitive, Ciprofloxacin-sensitive; MSCR: Methicillin-sensitive, Ciprofloxacin-resistant; MRCS: Methicillin-resistant, Ciprofloxacin-sensitive; MRCR: Methicillin-resistant, Ciprofloxacin-resistant; VS: Vancomycin-sensitive; VR: Vancomycin-resistant; CR: Ciprofloxacin-resistant.
[b] ND: not determined.

the initial residues chosen rather than the most potent peptide in the entire library.

Nevertheless, both strategies for deconvolution were useful in the identification of the two most active peptides described in this work. Using D-tryptophan (w) at position 1, based on iterative deconvolution data, and a combination of several residues in the remaining positions, based on positional scanning data, several CheA inhibitors were identified. Interestingly, both deconvolution strategies yielded similar results with respect to the key residues in certain positions, such as 2, 3, and 4, in which D-isoleucine (i), D-tyrosine (y), and D-isoleucine (i), respectively, were chosen for further deconvolution (Table 1; Figure 3). It is worth noticing that the overall chemical nature of the individual compounds identified through a solely iterative deconvolution process or a combination of the two processes is similar. These results therefore show that, in certain cases, a combination or parallel use of the two deconvolution processes can be useful for deconvoluting combinatorial libraries and can lead to the identification of several families of active sequences.

It is clear that the deconvolution of a combinatorial library, such as the one described here, is a complex process. Several factors are likely contributors to this complexity. First, the presence of closely related analogs, binding to the same site with significant affinity, could raise the apparent aggregate activity of the mixture being screened. Second, individual compounds in a combinatorial mixture could potentially bind and interact with the target at different sites, thereby raising the overall inhibitory activity of the mixture in an additive manner. Third, certain compounds in a mixture could have a synergistic effect, thereby drastically increasing the inhibitory activity. Fourth, certain compounds could have antagonistic effects relative to one another, thereby reducing the overall inhibitory activity of the mixture. One further complexity introduced during the deconvolution of a mixture-based combinatorial library is the potential decrease in the apparent

solubility of the active molecular species with an increase in its purity resulting from a decrease of number of individual compounds within a mixture.

The effects of the first two factors are apparent in our work. For example, when Ac-wixxxx-NH_2 was detected as the most active mixture in the CheA screen, with an activity level of ∼50% inhibition, the concentration of the final hit, Ac-wiyirr-NH_2 in the mixture was ∼1 nM (1/130321 of the mixture concentration). However, the apparent IC_{50} of Ac-wiyirr-NH_2 was ∼8 μM (Figures 2 and 3). In addition, if a single compound was responsible for the inhibition, then in each step of deconvolution, a 19-fold increase in potency should have been observed. In practice however, the highest increase in potency was ∼fivefold in deconvoluting Ac-wixxxx-NH_2 to Ac-wiv(y)xxx-NH_2. It is therefore clear that the overall activity of the Ac-wixxxx-NH_2 (apparent IC_{50} ∼125 μM) mixture was attributable to numerous individual peptides acting in an analogous, additive, and/or synergistic manner. If the analog effect was predominant, there were several thousands of peptides in the Ac-wixxxx-NH_2 mixture acting at the same site of CheA. If the additive effect was predominant, there could be up to several thousands of different inhibitor-binding sites on the target. It is possible that a combination of analog and additive effects was responsible for the inhibition potency of combinatorial mixtures observed during this work.

Finally, while ligands with nanomolar potency levels to various receptors and antibodies were identified in numerous studies using mixture-based combinatorial peptide libraries, only two cases of nanomolar inhibitory activity of peptides toward enzymes have been reported [25,26]. The micromolar activity levels of enzyme inhibitors, such as those described here, are probably indicative of a combination of several factors: (a) low affinity of these peptides for the enzyme of interest, (possibly due to insufficient peptide length); (b) high conformational flexibility of the peptide inhibitors; (c) high structural flexibility of the enzyme of interest (relative to receptors or antibodies). The use of combinatorial libraries built around scaffolds with less conformational flexibility could potentially lead to inhibitors with higher affinity, which in turn could lead to higher inhibitory activity.

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