A Parallel Synthetic Approach for the Analysis of Membrane Interactive Copolypeptides

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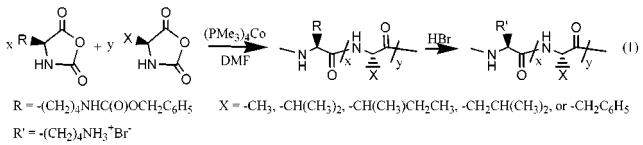
Parallel and combinatorial approaches have been shown to be highly effective for discovery and refinement of pharmaceutical compounds.[1] In particular these methods identify the underlying structure−activity relationships (SAR) useful for design of molecules with high activities and specificities.[2] In probing macromolecular interactions, such as between proteins and lipid membranes, elucidation of SAR is challenging since the molecular interactions can span relatively large distances (nm) and involve multiple sites. Small molecule libraries typically cannot be used to model such systems where cooperativity and macromolecular conformations are significant. Here we report a strategy for analysis of macromolecular interactions that capitalizes on the ability to readily prepare arrays of synthetic copolypeptides with well-defined chain lengths and compositions. Amphiphilic copolypeptides were synthesized in parallel and the resulting library was screened using a colorimetric assay to identify SAR involved in membrane interactions. The results of this study give insight into some of the fundamental characteristics of membrane active peptides and may lead to the design of improved antibiotics.

Nature has provided nearly all organisms with innate defense peptides (usually 10−30 residues) with broad ranging antimicrobial activities.[3,4] They are generally believed to kill microbes through membrane penetration and pore-formation.[5,6] These peptides vary widely in both sequence and composition, yet many are fairly simple, being composed primarily of cationic and hydrophobic residues.[2] Although there have been considerable studies on these materials, controversy remains as to their true mode or modes of action. For example, the importance of α-helix vs *β*-sheet favoring residues in enhancing efficacy remains uncertain.[2,7] One reason for this is the complexity and limited number of peptide samples that have been studied. Parallel approaches have not been utilized since diverse expression libraries of antimicrobial peptides are not feasible, and solid-phase synthesis is both cost-prohibitive and tedious for peptides of this size.[8] Furthermore, shorter pepides (<10 residues) are ineffective in these studies since they lack conformational stability and are unable to span lipid bilayers.[9]

Synthetic polypeptides offer many advantages for study of membrane interactive peptides. Seminal studies on the membrane activity of synthetic polypeptides were performed nearly 50 years ago.[10] While these studies showed that synthetic polypeptides and copolypeptides were able to bind to and disrupt cellular membranes, the large chain length distributions of these polymers and limited number of compositions studied prevented identification of any structure−function correlations. We recently developed techniques that allow the straightforward synthesis of block and random copolypeptides where both chain length and amino acid composition can be precisely controlled.[11] Fine adjustment of these parameters also leads to the ability to manipulate chain conformations. This system is well-suited for SAR studies since the copolypeptides are readily prepared, and are able to mimic the charge density, secondary structures, and amphipathy of larger peptides. Furthermore, simple liquid handling procedures make this methodology amenable to manual pipetting or robotic systems. Using these methods, we were able to generate approximately 500 polypeptides in less than 2 weeks time.

The design of this copolypeptide library was based on binary mixtures of amino acid components polymerized into statistically controlled sequences (eq 1). These "random" copolypeptides were



(1)

R = -$(CH_2)_4NHC(O)OCH_2C_6H_5$ X = -CH_3, -$CH(CH_3)_2$, -$CH(CH_3)CH_2CH_3$, -$CH_2CH(CH_3)_2$, or -$CH_2C_6H_5$

R' = -$(CH_2)_4NH_3^+Br^-$

composed of hydrophilic L-lysine residues plus one of the following hydrophobic amino acids: L-leucine, L-phenylalanine, L-isoleucine, L-valine, or L-alanine. These components were chosen so that the copolypeptides would mimic the cationic and amphiphilic nature of many natural antimicrobial peptides.[12] These amino acids were also selected based on their conformational tendencies. Leucine, phenylalanine, and alanine are all known to be α-helix favoring amino acids, while isoleucine and valine favor formation of *β*-structures.[13] Comparisons between these samples were expected to reveal the importance of chain conformation in polypeptide−membrane interactions. The parameters that were varied within each binary mixture were chain length, which ranged from 5 to 200 residues, and hydrophobic content, which ranged from 1 to 50 mol %. The simplicity of these samples, as compared to complex peptides containing many different amino acids, was expected to aid in the deconvolution and evaluation of chain length, hydrophobicity, and conformation effects.

This copolypeptide library was screened with a colorimetric assay[14] utilizing lipid vesicles composed of a diacetylenic surfactant (PDA)[15] mixed with a lipid of choice (e.g. DMPC, DMPG, or DMPE).[15] The diacetylenic units were then irradiated to form polydiacetylene chains, whose chromophores are highly sensitive to changes in membrane deformation.[16] The chromophores absorb in the visible, so changes in membrane structure are readily observed optically, and can be quantified spectroscopically. This assay has been used successfully for analysis of many types of membrane interactions, including binding of antimicrobial peptides.[16] The as-formed violet vesicles are typically observed to change color to orange or red upon interaction with these peptides.[17] In addition to identification of membrane-active

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(15) PDA = 10,12-pentacosadiynoic acid; DMPC = dimyristoylphosphatidylcholine; DMPE = dimyristoylphosphatidylethanolamine; DMPG = dimyristoylphosphatidyl-glycerol.
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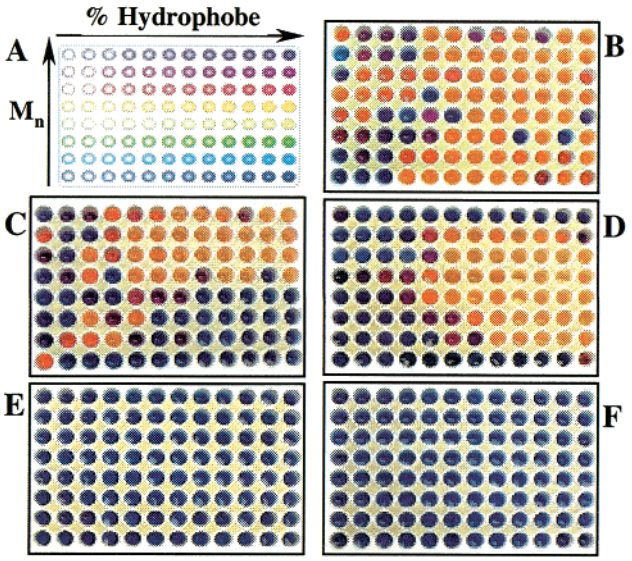

Figure 1. Copolypeptide library incubated with DMPC/PDA vesicles: (A) schematic layout; (B) Lys/Ala samples; (C) Lys/Phe samples; (D) Lys/Leu samples; (E) Lys/Ile samples; (F) Lys/Val samples. M_n = number average chain length. [copolypeptide] = 1.0 μM.

compounds, this assay allows analysis of lipid selectivity when different lipid mixtures are used. This feature is particularly useful in identifying compounds that interact strongly with microbes, but which should interact only weakly with mammalian cells, as these have significantly different cell wall lipid compositions.[18]

Incubation of the copolypeptide library with DMP(X)/PDA vesicles resulted in rapid (<20 min) colorimetric responses that varied with chain length, hydrophobe contents, and amino acid compositions (Figure 1).[19] The most reactive regions were generally seen to consist of samples with high hydrophobe contents and intermediate chain lengths, similar to natural antimicrobial peptides. The polypeptide libraries also displayed lipid selectivity in this assay. For example, some of the Lys/Leu compounds, when diluted, showed preferential reaction with DMPG/PDA and DMPE/PDA over DMPC/PDA vesicles (Figure 2). Hence we identified compounds (Figure 2, boxed) that show preference for microbial cell wall lipids[18] and are thus good candidates for more detailed analysis.

Examination of the entire plate also proved valuable, revealing many outlying, highly active areas that did not follow general trends (Figure 1b-d), illustrating the utility of this parallel approach for nonintuitive lead identification. The most striking result was that the PDA vesicles did not respond at all to copolypeptides containing β-favoring amino acids (Figure 1e,f), even though some of these are merely structural isomers of the colorimetrically active α-favoring copolypeptides (i.e. isoleucine versus leucine). These results suggest that chain conformation plays a key role in determining polypeptide activity.

To further investigate these interactions, we studied the mixture of copolypeptides with lipids using ^{31}P {^{1}H} NMR.[20] Surprisingly,

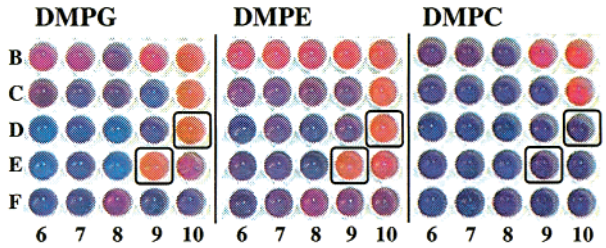

Figure 2. Selected region of the Lys/Leu library incubated with DMPG, DMPE, or DMPC lipid mixed with PDA. Boxes highlight selective samples. Axes refer to specific sample locations as defined in the Supporting Information. [copolypeptide] = 0.5 μM.

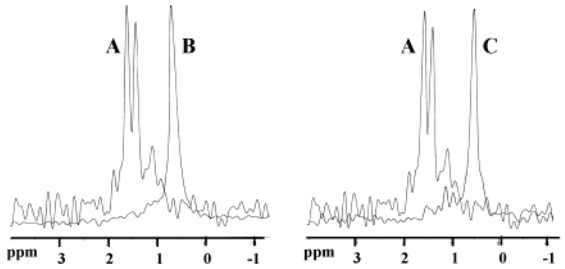

Figure 3. ^{31}P {^{1}H} NMR spectra of DMPC/decanoic acid vesicles: (A) 1 mM vesicles; (B) 1 mM vesicles + 1 μM Lys/Val sample D8; (C) 1 mM vesicles + 1 μM Lys/Leu sample D10.

these experiments showed that, in addition to the α-favoring samples, the β-favoring copolypeptides were also interacting with the phospholipid headgroups (Figure 3).[19] As further evidence of this membrane-binding activity of the β-favoring copolypeptides, it was found that pretreatment of PDA-vesicles with either Lys/Val or Lys/Ile samples followed by treatment of the vesicles with reactive Lys/Ala samples resulted in no color change.[19] Apparently, although binding of the β-favoring copolypeptides results in no colorimetric response, it is strong enough to prevent binding of the reactive helix-favoring samples.

Overall, this parallel synthetic method coupled with the optical assay provides a straightforward and efficient system for identifying important SAR in membrane disruption. We identified key compositions and chain lengths of leucine copolypeptides with high membrane reactivity that were also lipid selective. More significantly, these studies revealed key differences between the α- and β-favoring copolypeptides in membrane interactions. Ideally, this information can be used to develop potent antimicrobial, yet nonhemolytic peptide pharmaceuticals.

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Supporting Information Available: Details of library synthesis, PDA assay, and spectroscopic data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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