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RTD-1Mimic Containing γPNA Scaffold Exhibits Broad-Spectrum Antibacterial Activities

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Supporting Information

ABSTRACT: Macrocyclic peptides with multiple disulfide cross-linkages, such as those produced by plants and those found in nonhuman primates, as components of the innate immunity, hold great promise for molecular therapy because of their broad biological activities and high chemical, thermal, and enzymatic stability. However, for some, because of their intricate spatial arrangement and elaborate interstrand cross-linkages, they are difficult to prepare de novo in large quantities and high purity, due to the nonselective nature of disulfide-bond formation. We show that the disulfide bridges of RTD-1, a member of the θ-defensin subfamily, could be replaced with noncovalent Watson−Crick hydrogen bonds without significantly affecting its biological activities. The work provides a general strategy for engineering conformationally rigid, cyclic peptides without the need for disulfide-bond reinforcement.

Peptides and peptidomimetics could potentially be a rich source of pharmacophores for drug discovery because of the ease and flexibility of their synthesis and diverse sequence space that can be generated from a limited number of chemical building blocks; however, the main drawback is conformational flexibility. Because of their diminutive size and high degree of conformational freedom, they neither bind with high affinity nor selectivity, which has been the Achilles' heel of peptide therapeutics. Plants and certain organisms, including nonhuman primates, however, have devised a creative solution to this problem by stitching linear peptides into cyclic[1] and knotted[2−5] structures. Such architectural transformations not only confer conformational rigidity but also chemical, thermal, and enzymatic stability to otherwise highly flexible and enzymatically labile molecular entities.[6,7] These post-translationally modified peptides are commonly referred to as "cyclotides",[7] for those produced by plants, and as "defensins",[8] for those found in primates as part of an innate immunity. The latter class is further divided into three subfamilies, α, β, and θ, based on the spacing and pairing of the six disulfide-bonded cysteines. One such example of the last subfamily is RTD-1, a cyclic peptide with three disulfide bonds recently isolated from the leukocytes of rhesus macaques[9] and baboons[10] (Chart 1A). Though humans do not naturally produce this peptide, due to a premature stop-codon occurrence which took place ∼9 million years ago, a chemically synthesized version of this peptide called "retrocyclin"[11] has been made and, like the rhesus RTD-1 counterpart, has been shown to exhibit a broad spectrum of antibacterial,[9,12] antifungal,[9] and antiviral activities.[11,13,14] The discovery of these biologically active, cyclic peptides has spawned considerable interest in their development as therapeutics for treating fungal, bacterial, and viral infections as well as a number of chronic conditions including neuropathic pain.[15] While a number of these cyclic peptides have been chemically synthesized, they are generally difficult to prepare in large quantities and high purity needed for most clinical applications, especially those containing multiple and elaborate disulfide cross-linkages, due to the nonselective nature of disulfide-bond formation. As a proof-of-concept, we show that the disulfide bridges of RTD-1 could be replaced with noncovalent, Watson−Crick hydrogen bonds without significantly affecting its biological activities. The newly developed RTD-1 mimic could be easily prepared in large scale and high purity and is effective in killing both Gram-positive and -negative bacterial strains.

The structure of RTD-1 has been determined by NMR,[16] comprised of an extended β-hairpin reinforced by three disulfide bonds. Though its mechanism of action is not yet fully understood, studies of related members of this peptide family suggested that they confer their biological effects by interacting with the membrane of bacteria or pathogens.[18,19] The interaction causes perturbation in the membrane dynamics or potential, resulting in cell death or rendering the pathogens incapable of infecting the host cells. Based on this general mechanism of action, we surmised that it might be feasible to develop a synthetically simpler version of RTD-1, by replacing the disulfide bridges with noncovalent Watson−Crick hydrogen bonds, without significantly affecting its biological activities.

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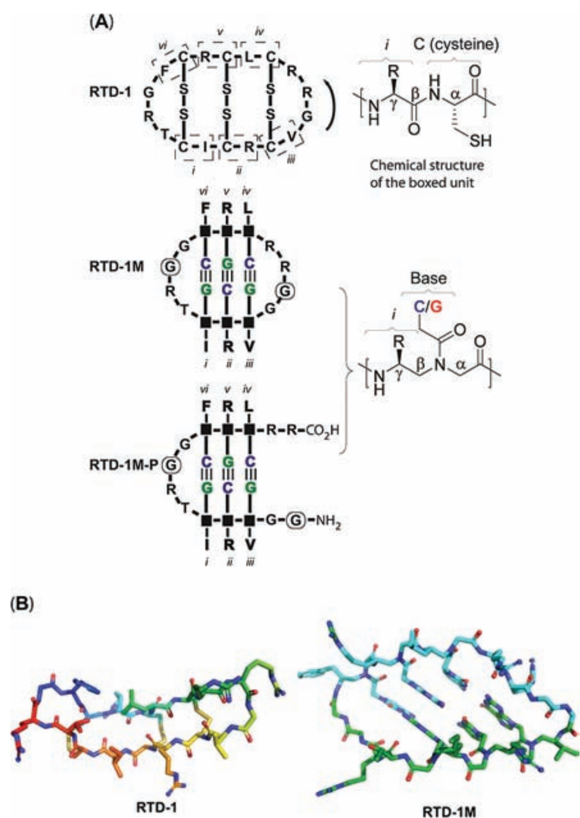
Chart 1RTD-1[a]



[a](A) Chemical composition of RTD-1, cyclic RTD-1M, and acyclic RTD-1M-P. Note that in RTD-1M and RTD-1M-P, the disulfide bonds are replaced with C-G base pairs. (B) NMR structure of RTD-1 as determined by Craik and coworkers[16] and energy minimized structure of RTD-1M using γPNA-γPNA duplex as a starting point.[17].

To test this hypothesis, we synthesized a cyclic RTD-1 mimic (RTD-1M) containing a mixture of natural L-amino acids and γ-peptide nucleic acid (γPNA)[20] building blocks—chiral derivatives of PNA with an amino acid side-chain installed at the γ-backbone position [Chart 1A and Chart S1, Supporting Information (SI)].[21] To preserve the chemical functionalities of the native RTD-1, we grafted the side chains of a selected set of amino acids onto the γ-backbone positions of PNA and replaced the cysteine side chain with cytosine (C) or guanine (G) nucleobase. The rest of the amino acid residues in the loop were kept intact except the glycine residues depicted in circles, which were inserted to provide an optimal loop-size for intramolecular hybridization (Chart 2S and Figures S1 and S2, SI). The acyclic precursor, RTD-1M-P, was prepared on solid-support according to a standard procedure[20] and cyclized postcleavage via intramolecular, template-directed ligation to give the desired RTD-1M (Figures S3–S6, SI).

Since there have been few reports of nucleic acid systems with three or fewer base pairs[22,23] and all of which contained entirely different backbone skeletons, it was not clear whether such a relatively short cyclic RTD-1M can maintain a stable, β-sheet-like conformation at physiological temperatures. To address this question, we performed circular dichroism (CD) and UV–vis spectroscopic analyses of RTD-1M and RTD-1M-P. Our result showed that both the cyclic and acyclic forms adopt a similar backbone conformation as that of the native RTD-1 (Figure 1), evidenced from the overlapping exciton

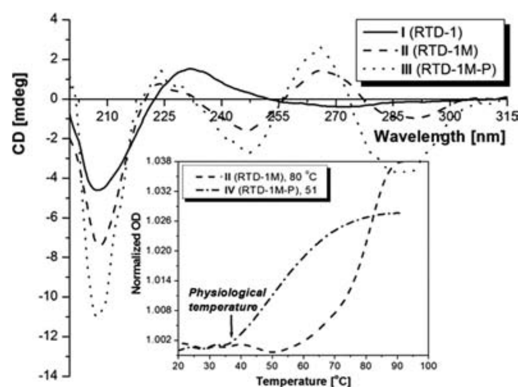


Figure 1. CD spectra of RTD-1, RTD-1M, and RTD-1M-P at 5 μM each in 10 mM sodium phosphate buffer (pH 7.4), recorded at 37 °C. Inset: UV-melting curves of the corresponding RTD-1M and RTD-1M-P in the same buffer.

coupling patterns in the 200–230 nm regions. This spectral regime has been previously assigned to the n–π* transition of the amide in the peptide backbone.[24] The CD spectrum in the 230–300 nm range is an indication of RTD-1M adopting a right-handed helix.[20] Since the duplex is relatively short, three base-pairs in length, it is expected to adopt an extended, β-sheet-like conformation but with a slight twist, as shown in Chart 1B. The UV-melting data revealed a significant increase in the thermal stability of the cyclic as compared to the hairpin precursor, with a T_m of ~80 °C for RTD-1M as compared to 51 °C for RTD-1M-P, corresponding to a net ΔT_m of +29 °C. These results together show that despite its small size, RTD-1M maintains a stable, β-sheet-like conformation at physiological temperatures.

Next, we assessed the antimicrobial activities of RTD-1, RTD-1M, and RTD-1M-P against *Escherichia coli*. The result showed that while RTD-1M and RTD-1M-P are progressively less potent, as compared to the native RTD-1; it is only by a small margin (Figure 2A). Like RTD-1, RTD-1M exhibits

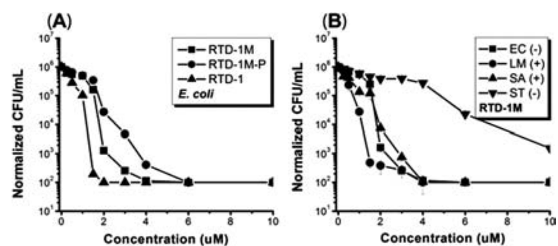


Figure 2. Microbicidal activities of (A) RTD-1M, RTD-1M-P, and RTD-1 against *E. coli* and (B) RTD-1M against a panel of Gram-positive and -negative bacterial strains. EC: *Escherichia coli*, LM: *Listeria monocytogenes*, SA: *Staphylococcus aureus*, and ST: *Salmonella typhimurium*. Each test organism was incubated with different concentrations of antimicrobial agents in 10 mM PIPES buffer (pH 7.4) containing 5 mM glucose and 0.03% TSB for 4 h at 37 °C in accordance with our published report.[9] The limit of detection was 1 colony per plate, corresponding to 1×10^2 colony forming units (CFU) per mL.

broad spectrum antimicrobial activities against both Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) and Gram-negative (*E. coli* ML 35 and *Salmonella typhimurium*) bacteria strains (Figure 2B).[9] The viability of *E. coli*, *S. aureus*, and *L. monocytogenes* was reduced by more than 99% after 4 h

incubation with 2 μM RTD-1M, whereas it is slightly less for *S. typhimurium*.

Consistent with the proposed mechanism of action that θ-defensins confer their bactericidal activity by interacting with and perturbing the membranes of the pathogens, treatment of bacteria with RTD-1M followed by brief incubation with SYTO-9 and propidium iodide revealed an intense red stain (Figure 3). SYTO-9 is a cell-permeable, green fluorescent probe that stains both live and dead bacteria, while propidium iodide is a cell-impermeant, red fluorescent probe that only stains cells with damaged membranes. The fact that all four bacterial strains treated with RTD-1M stained red, while the controls did not in the TRITC channel, indicates that RTD-1M is capable of disrupting the extracellular membrane of both Gram-positive and -negative bacterial strains. This finding was further corroborated by transmission electron microscopy (TEM), which revealed unusual extracellular morphologies for bacteria treated with RTD-1M, as compared to the controls. The effect is more pronounced in *E. coli* than in *S. aureus* (Figure 4). In the former, the membrane is dramatically distorted and broken-up, with cytoplasmic material leaking out; whereas in the latter, the outer membrane appeared to be swollen (or enlarged), coincided with aggregation and shedding of the extracellular membrane materials.

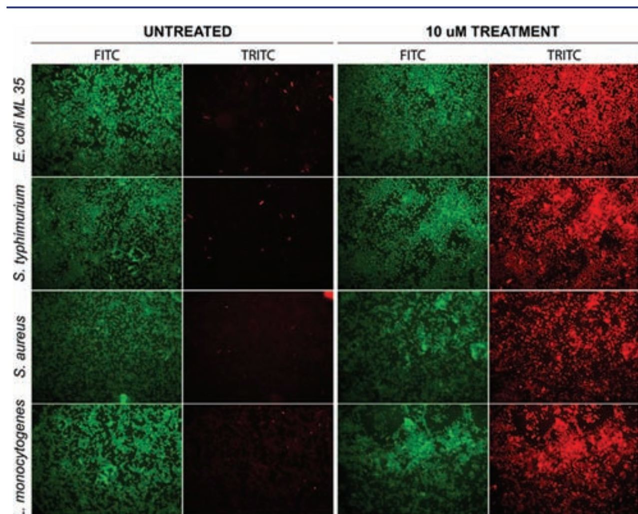


Figure 3. Fluorescent images of bacterial strains with and without RTD-1M treatment and after brief (15 min) staining with SYTO-9 and propidium iodide. The treatment was done with 10 μM RTD-1M for 4 h in 10 mM PIPES buffer (pH 7.4). The untreated samples were assayed the same way but without addition of RTD-1M.

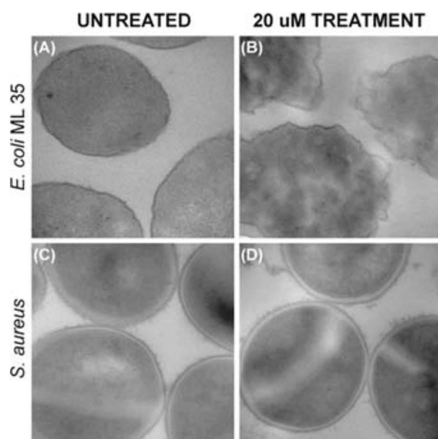


Figure 4. TEM images of *E. coli* and *S. aureus* with and without RTD-1M (20 μM for 2 h) treatments at 150 000× magnification.

Consistent with the previous observations with cyclotides[7] and defensins,[25] we noted that cyclic RTD-1M is significantly more stable in human serum than the linearized RTD-1, with a half-life of ~24 h as compared to ~2 min for the acyclic RTD-1 with Cys→Ser substitutions (Figures S2 and S8, SI). Hemolysis assay further revealed that RTD-1M is relatively nonlytic, as compared to most antimicrobial peptides found in nature.[26] At 250 μM, the highest concentration examined, less than 3.4% lytic activity was observed against human red blood cells (Table S1, SI). This result shows that like RTD-1, RTD-1M is cytoselective.

In summary, we have shown that a cyclic RTD-1 mimic containing natural L-amino acids and γPNA building blocks exhibits broad spectrum antimicrobial activities. Like the other members of the θ-defensin family, RTD-1M exerts its biological effect by interacting with and disrupting the cell membrane of bacteria. RTD-1M can be readily prepared on solid-support and cyclized postcleavage via intramolecular, template-directed ligation. Because of its relatively small size, such a cyclic molecule could be prepared in large quantities via modular, solution-phase methodology. Though standard, CDI-mediated coupling reaction was employed in the ring closure step in this study as a proof-of-concept, other coupling reactions including native chemical ligation,[27] Huisgen [3 + 2] cycloaddition,[28] and aziridine-mediated (4-component) condensation[29] could also be employed which may be operationally simpler and more efficient because of the high degree of orthogonality of these reactions. Likewise, the potency of RTD-1M could be further improved by modifications of the amino acid side chains, ring size, or incorporation of chemical moieties that are known to facilitate cell membrane anchoring, such as the alky tail of daptomycin,[30,31] or disrupting reagents, such as those inherent in magainin 2 and melittin.[26] While a large variety of classes of antibiotics have been developed and shown to be effective in combating bacteria,[32] almost invariably they succumb to resistance, rendering them ineffective over a prolonged treatment due to the rapid rate of genetic mutations of these pathogens. θ-Defensins and their synthetic analogues, such as RTD-1M, that confer their biological activities by interacting with and perturbing the cell membrane of bacteria or pathogens are less likely to develop resistance, as compared to those that target the genetic materials, because the cell membrane is under less selective pressure to evolve. Overall, the work provides a rational approach to controlling the spatial arrangement of peptides and construction of conformationally rigid, cyclic peptides without the need for covalent-bond reinforcement.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Monomer and oligomer synthesis protocols, HPLC, and MALDI-TOF spectra of RTD-1M-P and RTD-1M, UV-melting curves of various RTD-1M analogues with different loop sizes, results of enzymatic digestion and hemolysis assays, and other experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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