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Gramicidin S and polymyxins: the revival of cationic cyclic peptide antibiotics

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Abstract Gramicidin S and polymyxins are small cationic cyclic peptides and act as potent antibiotics against Gram-negative and Gram-positive bacteria by perturbing integrity of the bacterial membranes. Screening of a natural antibiotics library with bacterial membrane vesicles identified gramicidin S as an inhibitor of cytochrome *bd* quinol oxidase and an alternative NADH dehydrogenase (NDH-2) and polymyxin B as an inhibitor of NDH-2 and malate: quinone oxidoreductase. Our studies showed that cationic cyclic peptide antibiotics have novel molecular targets in the membrane and interfere ligand binding on the hydrophobic surface of enzymes. Improvement of the toxicity and optimization of the structures and clinical uses are urgently needed for their effective application in combating drug-resistant bacteria.

Keywords Bacterial membrane · Cationic peptide antibiotics · Drug target · Gramicidin S · Polymyxin · Respiratory enzymes

Introduction

The emergence of drug-resistant strains of major pathogenic bacteria such as *Staphylococcus aureus* is an increasingly serious public health concern [1]. To evade bacterial drug resistance mechanisms, new effective chemotherapeutic agents, which have novel mechanisms of action as well as different cellular targets compared with conventional antibiotics, need to be developed [2]. The shortage of new antibiotics to combat multidrug-resistant (MDR) strains has led to a renewed interest in polymyxins [3–7]. Polymyxins are active against MDR Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Enterobacter* species [3, 8]. Early reports described high incidences of nephrotoxocity and neurotoxicity during polymyxin therapy [9], and the use of polymyxins was replaced in the 1970s by antibiotics considered to be less toxic. However, recent studies showed that polymyxins have acceptable effectiveness and are considerably less toxic than originally reported [3].

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Cationic amphiphilic peptide antibiotics as a defense system

Short cationic amphiphilic peptides form part of the biological defense system of a broad range of organisms [10]. Cationic amphiphilic peptides are used by microorganisms to suppress the growth of competitors in the same ecological niche, while similar peptides form part of the innate immune defenses of higher organisms. Membrane lipid bilayer, rather than cellular proteins, is considered the primary target of these peptides. Interestingly, in mammals, many host defense peptides have additional chemokine-like and immunomodulatory activities [10, 11]. Unlike conventional antibiotics, the acquisition of resistance against antimicrobial peptides is surprisingly rare. Naturally occurring cationic amphiphilic peptides have retained their antimicrobial activity for millions of years, and may hold promise as broad-spectrum

antibiotics against the rapidly growing numbers of antibiotic-resistant microorganisms.

Gramicidins

The soil bacterium *Aneurinibacillus migulanus* (formerly known as *Bacillus brevis*) synthesizes nonribosomally and secretes the antibiotic tyrothricin, a mixture of linear pentadecapeptides (gramicidins A, B, and C) and backbone-cyclized cationic decapeptides [gramicidin S (GS) and tyrocidins [12, 13]] (Fig. 1). The primary structure of GS is [cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$]. GS consists of an antiparallel β-sheet structure supported by two type II′ β-turns and is amphiphilic, with two charged Orn side chains and two D-Phe rings projecting from one side of the molecule and four hydrophobic Val and Leu side chains projecting from the other [12–14]. GS is an extremely powerful antibiotic drug against a broad spectrum of both Gram-negative and Gram-positive bacteria with the minimum inhibitory concentration of 3–11 μM [15, 16]. Regrettably, GS is very hemolytic, which presently restricts its use to topical applications [13, 14]. GS is also effective against several pathogenic fungi, and Otoguro et al. [17] reported nematocidal activities of GS [the 50% inhibitory concentration (IC_{50}) = 0.08 μM] and polymyxins (IC_{50} = 0.8 μM) against the pine wood nematode *Bursaphelenchus lignicolus*. It was found later that GS was ineffective against the root-knot nematode *Meloidogyne incognita* even at 82 μM [18]. Effects and molecular targets of GS and polymyxins remain to be studied in lower eukaryotes.

Although the mechanism of antibacterial activity by GS is not completely understood, the primary mode of action is generally assumed to be the perturbation of lipid packing, resulting in destruction of the membrane integrity and enhancement of the permeability of the lipid bilayer of the bacterial cytoplasmic membrane [19–22]. Upon partitioning into lipid bilayers, GS displaces lipid molecules in the leaflet. Therefore, the accumulation of significant amounts of GS in a membrane is incompatible with the maintenance of a stable bilayer structure [23].

In contrast to GS, the structure of linear gramicidins like gramicidin A is an unconventional β-helix (6.3 amino acid residues per turn) with the alternating L- and D-amino acid composition except for position 2 (Gly). All side chains point outward, and linear gramicidins form N-to-N termini dimeric ion channels [24], which selectively transport alkaline metal cations and protons across the lipid bilayer [25].

Polymyxins

Polymyxins are an old class of cationic peptide antibiotics, and the emergence of MDR Gram-negative bacteria has led to the revival of polymyxins for salvage therapy [3, 4]. Polymyxins are pentabasic decapeptide antibiotics containing a cycloheptapeptide ring with a C9 or C10 fatty acid chain [6-methyl-octanoic acid (polymyxin B_1, Polymyxin E_1); 6-methyl-heptanoic acid (polymyxin B_2, polymyxin E_2)] through an α-amide linkage (Fig. 1) and nonribosomally synthesized in *Bacillus polymyxa* [26]. The target of antimicrobial activity is assumed to be the bacterial membrane. Cationic polypeptides bind to anionic lipopolysaccharide (LPS) molecules in the outer membrane of the Gram-negative bacteria, leading to a local disturbance of the membrane, which then causes an increase in the permeability [27, 28]. The polymyxin-mediated killing effect on the Gram-negative bacteria takes place prior to the increase in the membrane permeability, supporting a multi-hit hypothesis [29].

Screening of a natural antibiotics library

In order to identify new inhibitors for alternative respiratory enzymes (i.e., enzymes that are not present in human mitochondria), we screened the Kitasato Institute for Life Sciences Chemical Library [30] with the following enzymes: cytochrome *bd* quinol oxidase from *Escherichia coli* [31, 32], bacterial cyanide-insensitive oxidase (CIO, a variant of cytochrome *bd*) and a single-subunit NADH dehydrogenase (NDH-2) from the acetic acid bacterium

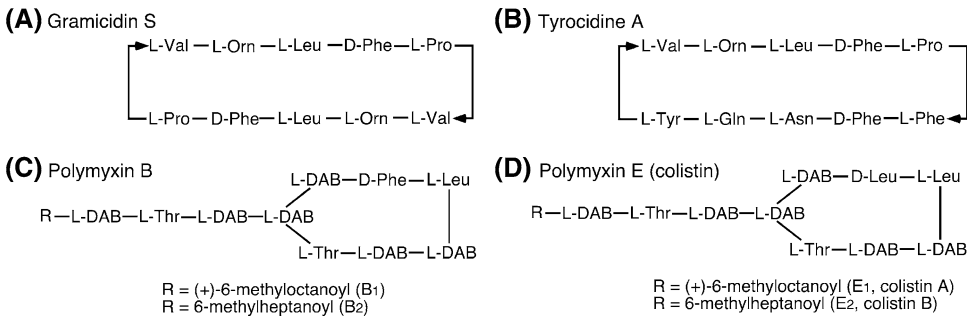

Fig. 1 Structures of gramicidins, tyrocidine A, and polymyxins. Tyrocidin B has Trp at position 6. *L-DAB* L-α-γ-diaminobutyric acid

Gluconobacter oxydans [33, 34], and NDH-2 and malate: quinone oxidoreductase (MQO) from *Mycobacterium smegmatis* [35] and *Pseudomonas aeruginosa* [36]. Cytochrome *bd* and CIO are widely distributed among bacteria and play an important role in microaerophilic respiration and protection against oxygen stress and in the survival and adaptation of pathogenic bacteria [37–39]. NDH-2s were shown to be crucial for the adaptation of *M. tuberculosis* [39] and malaria parasites [40, 41], but their specific inhibitors are rare [42]. Therefore, inhibitors of quinol oxidases [31–33, 43] and NDH-2 [34, 35, 40, 41, 44] are promising new chemotherapeutics.

Library screening identified GS as a mixed-type inhibitor of *E. coli* cytochrome *bd*, while gramicidin D (the naturally produced mixture of gramicidins A, B, and C of ~80% A, 5% B, and 15%C) [45] did not show inhibitory activity [31]. The IC_{50} of GS (3.5 μM) was comparable to the IC_{50} of the known quinol oxidation site inhibitors, 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO; 1 μM) and antimycin A (5 μM), and to the IC_{50} (9 μM) for the aerobic growth of *E. coli* cells. Cytochrome *bo* quinol oxidase and NDH-2 were tenfold less sensitive to GS than cytochrome *bd* oxidase, and succinate dehydrogenase (Complex II) was totally unaffected by GS. Notably, GS had a stimulatory effect at low concentrations by increasing the apparent V_{max} value of cytochrome *bd* quinol oxidase [31, 43].

From screening with *G. oxydans* NDH-2, we identified GS and scopafungin as potent inhibitors and found the moderate inhibitory activity with polymyxin B [34]. GS serves as a noncompetitive inhibitor for NADH oxidation and a competitive inhibitor for quinone (Q) reduction. Furthermore, GS shows inhibitory activity towards NDH-2 of *M. smegmatis*, and the IC_{50} value of GS (2 μM) is significantly lower than that of trifluoroperazine (12 μM) for *M. tuberculosis* NDH-2 [44]. Recent screening of the natural antibiotics library revealed that that polymyxin B acts as a quinone reduction site inhibitor of *M. smegmatis* NDH-2 and MQO [35].

New mechanism for the action of cationic cyclic peptide antibiotics

GS and polymyxins are structurally unrelated cationic cyclic peptides, but their targets for antimicrobial activity are assumed to be the bacterial membranes. GS acts on the lipid bilayer of the cytoplasmic membrane and enhances the permeability by destroying membrane integrity [19–22]. Polymyxins bind to LPS in the outer membrane of the Gram-negative bacteria and lead to a local disturbance of the outer membrane which results in increased permeability of the cytoplasmic membrane [45–47]. Polymyxins can exhibit antibiotic activity towards mycobacterial species, which have the micolic acid-based cell wall in place of LPS [48, 49]. Thus, a universal mechanism of GS and polymyxins for their antibacterial activities is the direct or indirect destruction of the bacterial cytoplasmic membranes [19–22, 45–47].

Gramicidins have been shown to inhibit eukaryotic P-type ATPases. Kasamo [50] observed the inhibitory effect of GS (IC_{50} = 24 μM) and tyrocidine on plasma membrane Mg^{2+}/K^{+}-ATPase from tobacco leaves. Gramicidin D (linear gramicidins) was less effective than GS. Zhao and Dhalla [51] reported that GS inhibited rat heart plasma membrane Ca^{2+}-ATPase uncompetitively (IC_{50} = 3 μM) and sarcoplasmic reticulum Ca^{2+}-ATPase in a mixed-type manner (IC_{50} = 6 μM). Iglesias and Rega [52] found that GS inhibited Ca^{2+}-ATPase of human red-cell membranes by lowering the maximum velocity of the high affinity component and the apparent affinity of the low-affinity component. Reversal of the inhibitory effect of GS by liposomes suggests that GS acts on the hydrophobic domain of Ca^{2+}-ATPase. It was found recently that gramicidin A directly inhibits ATP hydrolysis of Na^{+}/K^{+}-ATPase (IC_{50} = 8 μM) from porcine cerebral cortex in the mixed-type manner while GS (IC_{50} = 41 μM) is less effective against Na^{+}/K^{+}-ATPase [53]. Notably, a mixture of linear gramicidins inhibits RNA synthesis by the purified *A. migulanus* RNA polymerase at high concentrations (50% inhibition at 54 μM), by interfering binding to DNA [54]. These observations indicate that GS and other peptide antibiotics interact with distinct targets other than the lipid bilayers.

By taking the advantages in structural variations of natural compounds and respiratory enzymes from different species, we carried out matrix screening of the natural antibiotics library with bacterial membranes. We demonstrated that the library is a potential source of species-specific novel inhibitors of respiratory enzymes [31–36, 43]. Cationic cyclic peptide antibiotics like GS and polymyxins would be accumulated in bacterial cytoplasmic membranes and then reach to the quinone-binding sites in the hydrophobic domains of peripheral or intrinsic membrane proteins (Fig. 2). So far, only membrane-bound respiratory enzymes [31–36, 43] and P-type ATPases [50–52] have been identified as molecular targets of GS and polymyxins. Penetration of peptide antibiotics across the bacterial membranes, and the identification of soluble molecular targets need to be examined in future studies. Considering their poor solubility in aqueous solutions, the upper limits of IC_{50}s for chemotherapeutically important drug candidates are the μM concentration. Thus, our findings provide new insights for the molecular design and development of cationic cyclic peptide antibiotics targeting to bacterial membrane proteins like respiratory enzymes.

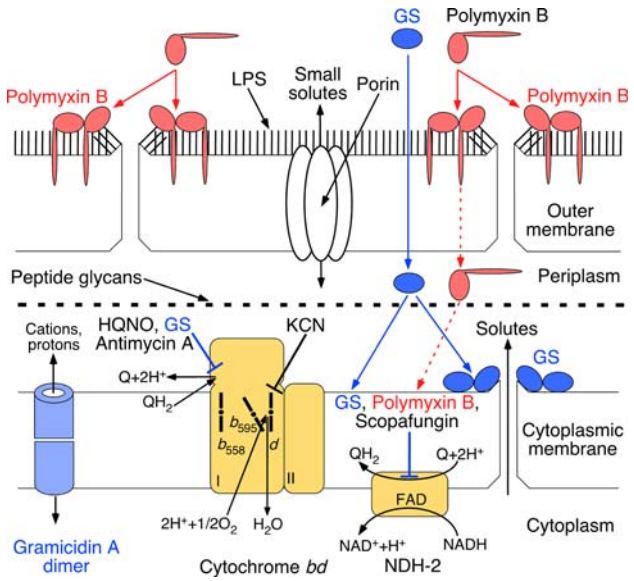

Fig. 2 Interactions of gramicidin S (*GS*), gramicidin A, and polymyxin B in the cell surface of the Gram-negative bacteria. See details in text

Concluding remarks and perspectives

Recent studies have shown that structural analogs of GS [16, 55–57], tyrocidine [58], and polymyxin [59] can be designed with markedly reduced hemolytic activity and enhanced microbial activity, suggesting that cationic cyclic peptide antibiotics could be used as potent oral or injectable broad-spectrum antibiotics. In addition to the chemical synthesis approach, as shown for gramicidin S synthase [60], computational structure-based redesign of the nonribosomal peptide synthase is an alternative strategy to facilitate making new cyclic peptide antibiotics in a large quantity. Previous studies on polymyxins have likely been insufficient to abandon their clinical uses. Randomized controlled trials, the determination of the pharmacokinetic/pharmacodynamic properties, the development of improved formulations [61], dosage optimization, and the evaluation of toxicity of peptide antibiotics, are all urgently needed for treatments of infections with MDR bacterial strains. Continuing efforts to identify antibiotics with new targets and mechanisms of action, as well as careful examination of their clinical application, will lead to the development of new chemotherapeutics against drug-resistant bacteria. Further, tyrocidin A ($IC_{50} = 0.6$ nM) [62] and tryptophan-*N*-formylated gramicidin A (a non-hemolytic derivative with $IC_{50} = 2.6$ nM) [63, 64] have been shown to be potent antimalarial agents for the human malaria parasite *Plasmodium falciparum*. Application of peptide antibiotics to parasitic protists and other infectious disease needs to be examined.

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