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REVIEW

New Aspects of Protein Biosynthesis Inhibition by Proline-Rich Antimicrobial Peptides

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Abstract—Proline-rich antimicrobial peptides (PrAMPs) are promising compounds for overcoming antibiotic resistance, one of the global health threats, and stand out from other types of AMPs by their high safety profile. The main cellular target of PrAMPs, like most modern antibiotics, is the conservative cellular structure – the ribosome. PrAMPs bind in the ribosomal tunnel, forming multiple interactions with nucleotides of 23S rRNA, and are divided into two classes depending on their mechanism of action: inhibition of elongation or termination. The N-terminal part of the peptides, which is important for the activity of class I peptides, extends into the A-site pocket, preventing the binding of aminoacyl-tRNA. A new family of PrAMPs, rumicidins, was discovered using genomic search methods. Its representatives have the longest N-terminal part, as well as a unique pair of amino acids Trp23 and Phe24 at the C-terminus. The Trp-Phe dyad forms a spacer at the constriction site of the ribosomal tunnel, stabilizing the binding and leading to increased antibacterial activity. New structural studies of the class I peptide Bac5 have demonstrated its ability to disrupt the correct positioning of the CCA-end of the P-site tRNA in the peptidyltransferase center of the ribosome, which can affect the assembly of functional initiation complexes. Class II PrAMPs, according to new data, have additional binding sites on the ribosome and have a complex effect on the bacterial cell: they disrupt the termination of protein synthesis, block the cellular ribosome release system, prevent the correct assembly of the 50S ribosomal subunits, and, possibly, affect the first stage of translocation. Recent studies expand our understanding of the antimicrobial activity of PrAMPs and contribute to the creation of future therapeutic drugs based on AMPs.

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INTRODUCTION

Antimicrobial peptides (AMPs) are genetically encoded components of an organism's innate immune system [1]. Although AMPs are synthesized by the representatives of all kingdoms of life, the primary sources of known AMPs are animals (78%), bacteria (12%), and plants (8%) (Fig. 1). According to their name, most AMPs target bacteria and fungi, though some also exhibit antiviral, antiparasitic, and antitumor properties [2]. For potential therapeutic applications, animal-derived AMPs are the most promising [3]. Pathogens develop resistance to AMPs far less readily than to low-molecular-weight antibiotics, and the genetic barriers are higher, impeding the horizontal transfer of resistance gene between bacterial species [4, 5].

AMPs are structurally diverse and target both membranes, disrupting their barrier function, and intracellular components, inhibiting vital processes such as DNA repair, replication, transcription, translation, protein folding, and cell division [2]. A recent

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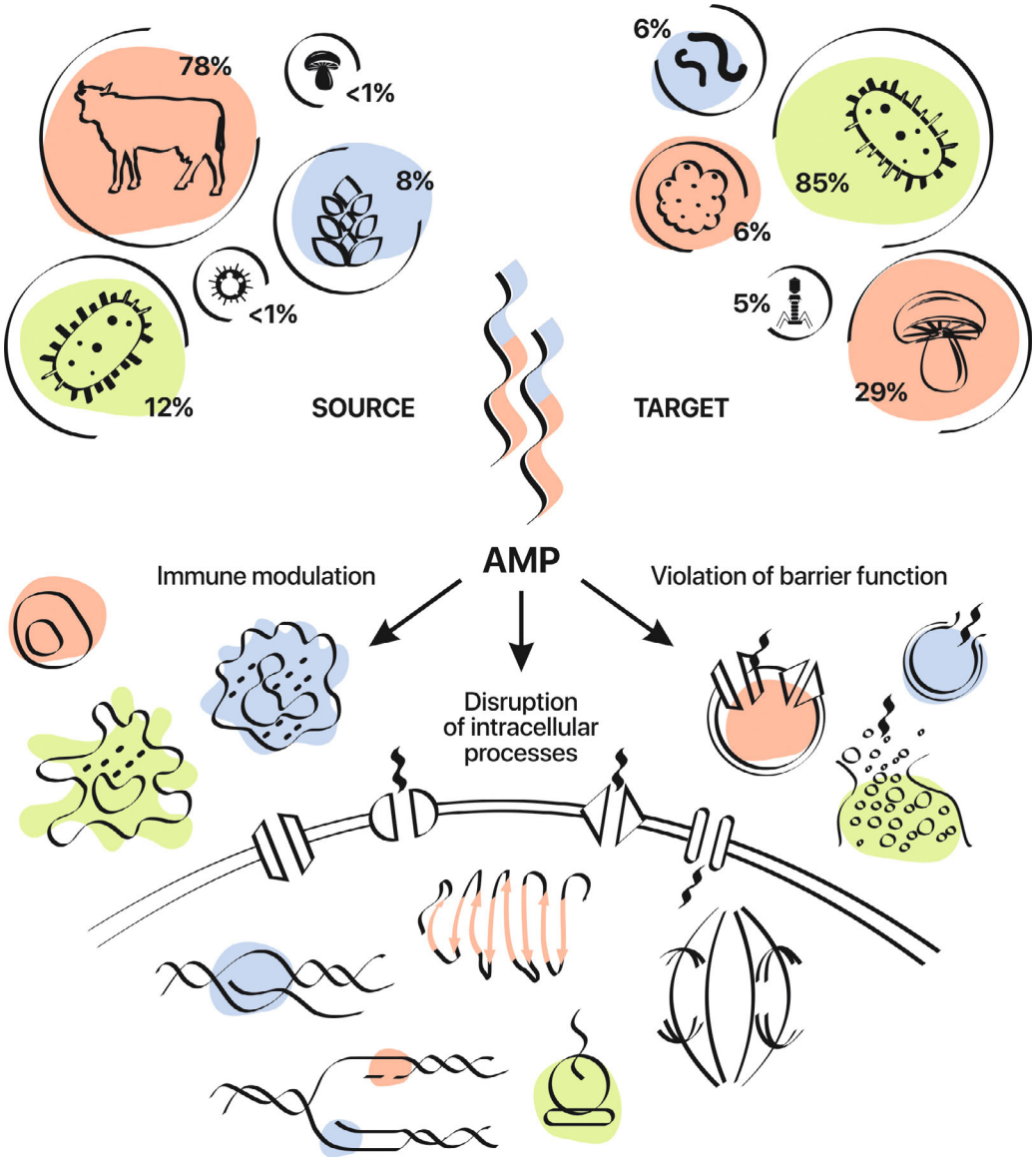

Fig. 1. Antimicrobial peptides (AMPs): sources, targets, and mechanisms of action (numerical values taken from the Antimicrobial Peptide Database APD; https://aps.unmc.edu/ as of January 2025).

discovery has revealed the ability of polyproline AMPs to disrupt bacterial biofilms [6]. The antibacterial mechanism of AMPs is often multifaceted. For instance, amphibian AMPs can not only form pores in bacterial membranes but also block membrane transport by affecting the expression and assembly of membrane proteins, inhibit efflux pump activity, disrupt energy metabolism by targeting ATP synthase, and inhibit function of FtsZ, a GTPase critical for cell division that has no direct analog in eukaryotic cells [7, 8]. Beyond direct elimination of infectious agents, AMPs can also modulate the host immune response by attracting and activating immune cells, which enhance pathogen destruction and inflammation control [9].

Most AMPs function by compromising the integrity of bacterial membranes, demonstrating activity against both Gram-negative and Gram-positive bacteria [10, 11]. However, these membranolytic properties are often associated with a poor safety profile for injectable substances, so they are primarily considered for topical use [12]. In contrast, certain classes of AMPs can enter bacterial cells non-destructively through porins to inhibit intracellular targets. This mode of action makes them more promising as lead compounds for systemic therapy [13, 14].

MAIN CHARACTERISTICS OF PROLINE-RICH ANTIMICROBIAL PEPTIDES

Proline-rich antimicrobial peptides (PrAMPs) efficiently penetrate bacterial cells without compromising

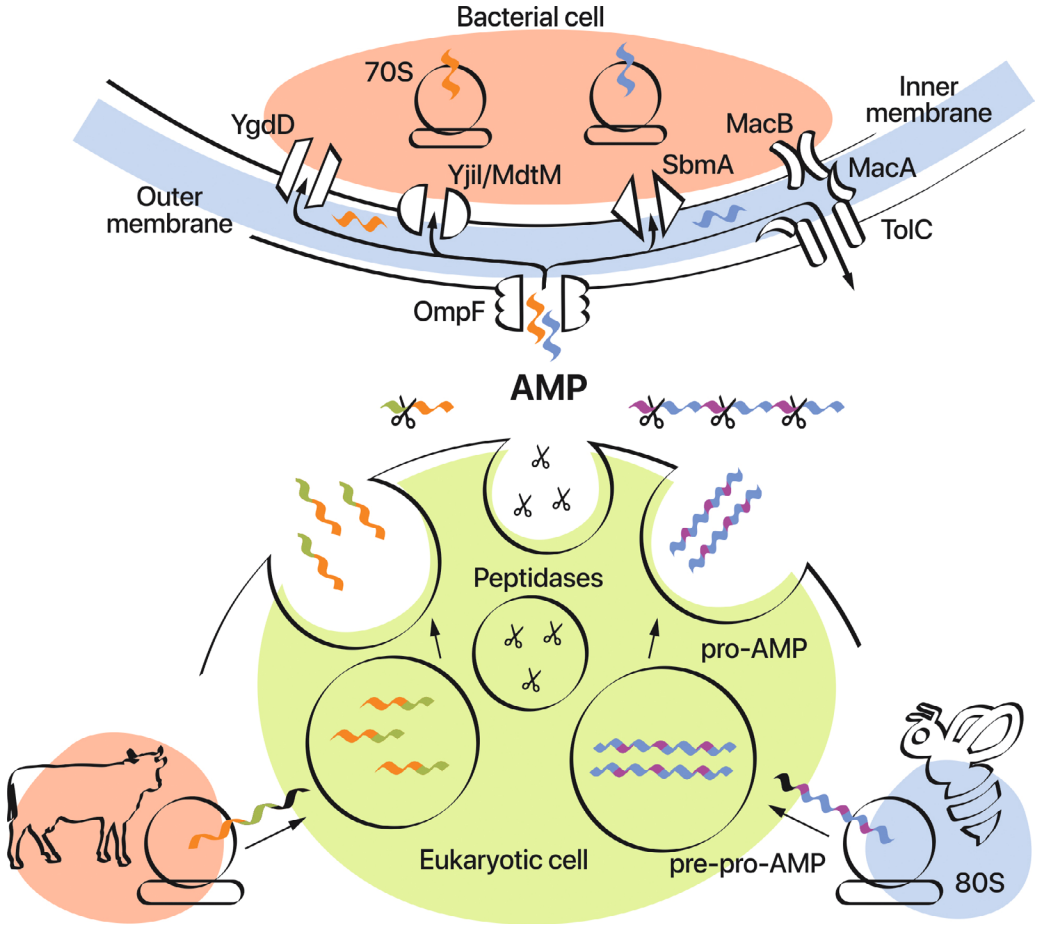

Fig. 2. Proline-rich peptides: synthesis and mechanisms of penetration into bacterial cells. PrAMPs are synthesized on the eukaryotic 80S ribosomes as inactive prepropeptides (pre-pro-AMP). The signal pre-sequence is cleaved off after directing the immature propeptide (pro-AMP) to large granules. In the extracellular space, pro-AMP is activated by peptidases and translocated into periplasmic space via the OmpF porin. Partial export of the peptide from the periplasmic space to the external environment can be carried out by the MacAB-TolC efflux pump. Transport of PrAMPs through the inner membrane into the cytoplasm is mediated by the proteins SbmA, YjiL/MdtM, and YgdD. Once inside the cytoplasm, PrAMPs interact with the target, prokaryotic 70S ribosome.

membrane integrity. They are characterized by a proline content exceeding 25%, a net positive charge, antimicrobial activity, and a mechanism of action that involves binding intracellular targets like the DnaK chaperone or 70S ribosome. Structurally, most PrAMPs contain a characteristic PRP motif, though this is not a strict requirement [15, 16]. Beyond their primary antibacterial role, many PrAMPs also exhibit antifungal activity [17]. Furthermore, some representatives of this class demonstrate immunomodulatory functions, such as enhancing chemotaxis and differentiation of dendritic cells, activating adaptive immune responses, and suppressing the release of pro-inflammatory mediators [18, 19]. The functional repertoire of these peptides may be even broader for those that incorporate additional domains alongside the proline-rich regions, such as cysteine-rich or acidic amino acid-rich domains, potentially leading to a wider spectrum of biological activity [15].

PrAMPs are predominantly found in arthropods, such as beetles, bees, wasps, flies and crabs, and in mammals, like sheep, cows, pigs, goats, dolphins. To date, only a single PrAMP has been identified in a plant (rapeseed) [15, 20]. The range of methods for discovering these peptides has expended significantly. The first PrAMP, apidaecin (Api), was identified after injecting honeybees with a sublethal dose of *Escherichia coli* and isolating the induced peptide chromatographically [21]. In contrast, modern discovery relies on bioinformatic analysis of whole-genome sequencing data, an approach that has recently led to the identification of new insect [22] and cetacean PrAMPs [13], and an entire family of proline-rich cathelicidins from ruminants, named rumicidins (Rum) [23]. Notably, humans and other primates do not produce PrAMPs [24], making the therapeutic prospect for supplementing this gap in the innate immune defense particularly attractive. Additionally,

PrAMPs exhibit high stability against proteolytic degradation, conferred by their increased proline content, and demonstrate no toxicity toward mammalian cell lines [25].

In animals, PrAMPs are synthesized by eukaryotic ribosomes as inactive precursors known as prepropeptides. The pre-region is a signal peptide that directs the immature PrAMP to large granules and is subsequently cleaved off [26, 27] (Fig. 2). This biosynthetic pathway ensures protection of the host's own ribosomes and ability to store antimicrobial components, enabling a rapid response to bacterial attack. The remaining propeptide consists of an inactivating pro-sequence attached to the AMP. In mammalian PrAMPs [28, 29] and in the fruit fly's drosocin [30] the pro-sequence is linked to a single antimicrobial peptide. The propeptides of most insect PrAMPs contain multiple, nearly identical AMP isoforms separated by short, conserved oligopeptide spacers [21, 31]. Final activation occurs through proteolytic cleavage, which releases the mature PrAMP. The active peptide is then transported to the extracellular space or into phagosomes to exert its action on bacterial target [27, 32].

Many PrAMPs at their minimum inhibitory concentration (MIC) (Table 1), do not permeabilize the bacterial membrane, but instead inhibit intracellular targets after passing through it [23, 33, 34]. Current data support a multi-stage penetration model of PrAMPs into the Gram-negative bacterial cells (Fig. 2). PrAMP electrostatically interacts with the outer membrane. The peptide is then translocated into the periplasmic space via the OmpF porin. Notably, a portion of the peptide may be exported to the external environment by the MacAB-TolC efflux pump [23]. Further transport of the peptide through the inner membrane into the cytoplasm is mainly carried out by the SbmA protein [35, 36], a permease family protein mediating the proton-dependent transfer of various antimicrobial agents [37, 38]. Subsequent research revealed an alternative non-lytic uptake pathway in the *E. coli ΔsbmA* cells without permeabilization and significant decrease in MIC for the PrAMPs such as several bactenecin 7 (Bac7) variants, and cetacean peptides Bal1 and Lip1 [13, 39]. To date, at least two additional inner membrane PrAMP transporters have been identified: YjiL/MdtM and YgdD. The role of YjiL/MdtM has been confirmed in the internalization of natural ruminant peptides Bac7(1-35) and rumicidin 1 (Rum-1), aphalin Tur1A, and the synthetically optimized oncocins (Onc) Onc18 and Onc112 [23, 37, 40]. YgdD mediates the uptake of the crab PrAMP arasin 1(1-23), and the insect peptides apidaecin 1b (Api-1b) and pyrrhocoricin (Pyr) [14, 41]. Functional characterization using individual knockout strains of each transporter established a clear hierarchy among these transporter systems. SbmA emerges as the primary importer, while YgdD and YjiL/MdtM serve as less important secondary transporters.

Table 1. Minimum inhibitory concentration (MIC) values of full-length and truncated PrAMPs against *E. coli* strain BW25113

Peptide	MIC, μM	References
Bac7(1-35)	3	[40]
Bac7(1-16)	8	[39]
Bac5(1-25)	1	[62]
Bac5(1-15)	64	[63]
Bac5(3-25)	4	[63]
Onc72	7	[37]
Onc112	2	[37]
Rum-1	2	[23]
Rum-1(1-22)	16	[23]
Rum-1(9-29)	8	[23]
Rum-1(11-29)	8	[23]
Tur1A	1.2	[40]
Tur1A(1-16)	4	[40]
Bal1	1	[13]
Lip1	1	[13]
Api88	0.4	[37]
Api137	2	[37]
Api137(1-17)	29	[37]

To date, two intracellular targets have been identified for PrAMPs, both of which ultimately disrupt the production of functional bacterial proteins. In 2000s, it was demonstrated that certain PrAMPs bind to the substrate-binding pocket of Hsp70 chaperone DnaK, thereby preventing proper protein folding [42, 43]. However, the observed sensitivity of DnaK-deficient lines to PrAMPs pointed to the existence of additional targets [44]. Subsequent studies revealed that the main target for PrAMPs is the bacterial ribosome [45], like for more than half of conventional low-molecular-weight antibiotics. The ribosome's central role in translation process and its structural conservation across bacterial species make it an ideal target for various antimicrobial agents [46].

Based on their mechanism of action, PrAMPs are categorized into two classes that target different

stages of the translation cycle. Class I peptides, which include oncocins and Bac7-like cathelicidins, bind to the initiation ribosomal complex and act during the elongation by sterically preventing binding of the first aminoacyl-tRNA in the A-site. In contrast, class II peptides, which include apidaecins and drosocin, bind to the ribosome after release of the synthesized polypeptide chain. They prevent dissociation of release factors RF1 and RF2 from the termination complex [20, 47, 48]. Both classes of PrAMPs bind within the nascent polypeptide exit tunnel (NPET) of the large ribosomal subunit. The tunnel is primarily formed by the negatively charged 23S rRNA, and peptide binding is stabilized by a network of hydrogen bonds, stacking interactions, and van der Waals forces [23, 40, 49-51]. This multi-point binding mechanism significantly reduces the risk of bacterial resistance developing through single-point mutations in the 23S rRNA [52, 53]. Structural data demonstrate that the majority of proline-rich peptides adopt a rigid, type II polyproline helix conformation [54]. This structure is critical for their function: it allows efficient translocation through the bacterial membranes without causing disruption of the lipid structure [55] and facilitates penetration into the NPET. Moreover, due to this conformation, PrAMPs poorly bind to the active site of proteases, which also contributes to their stability [56].

INTERACTION OF CLASS I PrAMPs WITH THE RIBOSOME

Class I PrAMPs bind to the NPET in reverse orientation relative to the growing polypeptide chain, i.e., N-terminus of the PrAMP is directed toward the peptidyltransferase center (PTC), and C-terminus extends deep into the NPET.

The N-terminal part of all class I peptides protrudes into the A-site tRNA binding pocket, however the number and composition of amino acids, as well as contacts formed with 23S rRNA, significantly vary between the arthropod and mammalian PrAMPs (Fig. 3). The insect peptides (Onc, Pyr, metalnikowin) are characterized by the short, conserved N-terminal part consisting of 2 amino acids, valine and aspartate, whose deletion disrupts binding to the bacterial ribosome, indicating key role of the N-terminal regions in antibacterial activity [49]. The N-terminal part of mammalian peptides (Bac7, PR-39, Tur1A) is extended to 4-5 amino acids and consists mainly of arginine residues. *In vitro* studies have shown that two N-terminal arginine residues are required for efficient penetration into bacterial cells, and their removal reduces antibacterial activity of the peptides [57]. Deletion of the first 4 amino acids almost completely inactivates the peptide, disrupting its interaction with the main target – the ribosome [52, 58]. Despite the increased length of N-terminus of the mammalian peptides, it does not extend deeper into the A-site pocket than the insect peptides. High arginine content in this region allows formation of a compact, positively charged loop, ideally suited in size and charge for anchoring interactions with the groove formed by the nucleotides C2452, A2453, and G2454 on one side and nucleotides U2493 and G2494 of 23S rRNA on the other, which is likely a characteristic feature of the mammalian PrAMPs aimed at increasing the strength of peptide-ribosome interaction [40, 52]. Thus, the N-terminal parts of the peptides are fundamentally important for antibacterial activity of all characterized representatives of class I PrAMPs, except for rumicidins, whose interaction with the ribosome will be discussed in detail below.

Central part of the class I PrAMPs, corresponding to the residues 3-11 of Onc and 6-14 of Bac7, is located mainly in the A-site cleft but also occupies a small area of the A-site pocket and the upper part of the tunnel. This is a highly conserved so-called consensus sequence (R/K)XX(R/Y)LPRPR containing the invariant PRP motif. Conformation of the consensus part of all structurally characterized peptides in the NPET of 70S ribosome complex is almost completely identical [23, 40, 49-52].

The residues Lys3 and Arg9, common to the insect PrAMPs (Onc112, metalnikowin, Pyr), form hydrogen bonds with A2453 and U2584, respectively, and Arg9 additionally participates in stacking interactions with C2610. The residues 5-7 of the insect PrAMPs correspond to the residues 8-10 of the mammalian PrAMPs. Tyr6 (Onc112, metalnikowin, Pyr) and the corresponding residue Arg9 of Bac7 and Tyr9 of Tur1A form stacking interactions with C2452 of 23S rRNA. The mammalian PrAMPs contain more arginine residues than the insect PrAMPs, establishing more stacking interactions in the NPET: Arg15 and Arg16 of the Tur1A peptide with U2586 and His69 of L4, respectively, and Arg12, Arg14, and Arg16 of the Bac7 peptide with C2610 (similar to Arg9 of insect PrAMPs), U2586, and A2062, respectively [40, 49, 51, 52].

Replacement of any residue in the Arg9-Leu10-Pro11 region of Bac7(1-16) drastically reduces its potential to inhibit bacterial protein synthesis and growth of *E. coli* cells, while substitutions in other regions have little effect on its activity [39]. Additionally, the use of deep mutational scanning, which allowed analysis of antibacterial potential of more than 600,000 variants of Bac7(1-23), demonstrated high importance of preserving the native sequence of Bac7 at positions 6 and 9-14, with the most pronounced invariance in the 9-11 segment [59].

The C-terminal residues of class I PrAMPs are considered non-conserved and less important for

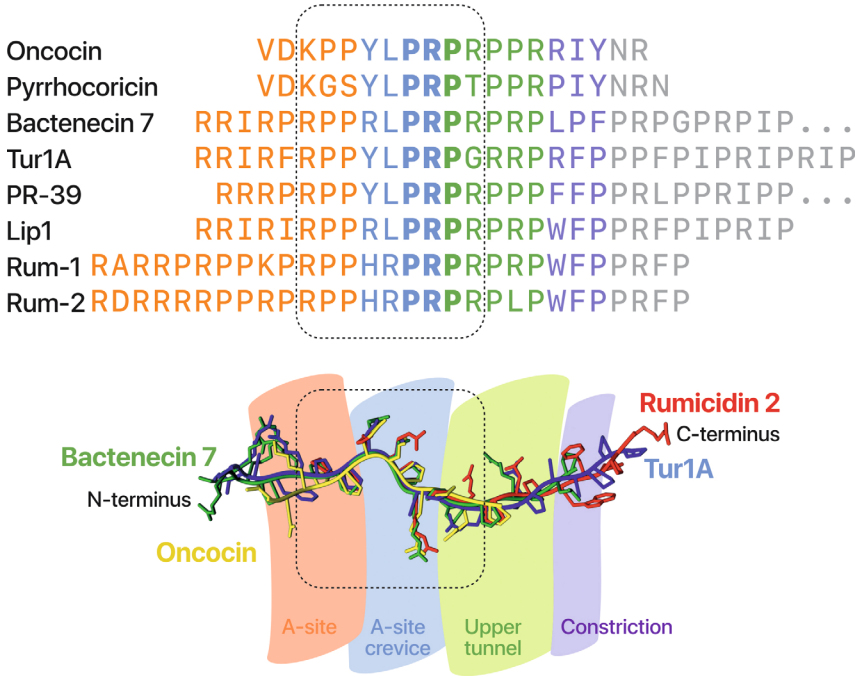

Fig. 3. Alignment of amino acid sequences and spatial models of class I PrAMPs (Rum-2, PDB ID: 9D89; Bac7, PDB ID: 5HAU; Onc112, PDB ID: 4Z8C; and Tur1A, PDB ID: 6FKR). The consensus region is indicated by the dashed line.

ribosome binding and inhibitory activity. In particular, the C-terminal region of Bac7 can be truncated to form peptides of 35, 23, and even 16 amino acids with minimal loss of antibacterial activity [60]. Similarly, antimicrobial activity of Tur1A was only slightly reduced when the peptide was truncated at the C-terminus to 16 amino acids [40]. In both cases, central part of the peptides, which ensures strong interaction with the ribosome, remained intact. Further truncation of Bac7 by just one amino acid to the 1-15 fragment [60], as well as deletion of 7 C-terminal residues from the initially shorter insect peptide to form the OncΔ7 peptide [51], resulted in complete loss of peptide activity, likely due to the critical proximity of the C-terminus to the consensus region. Additionally, it is worth noting that in the majority of available structures of the class I PrAMPs, C-terminal residues are poorly resolved [40, 49-52], which could indicate structural heterogeneity.

Against the background of uniformity of the sequences and structures of the above-described peptides, it is interesting to focus on the recently characterized representatives of the class I PrAMPs, which have a number of unique features.

Fixation of rumicidins in the constriction area of the nascent polypeptide exit tunnel. The search for cathelicidin genes in the whole-genome sequencing database of Cetartiodactyla using a fragment of the cathelicidin-like domain of the Bac7 precursor led to the discovery of *CATHL(3L2/8)*-like genes encoding an entire family of relatively short (28–30 amino acids) ruminant PrAMPs, named rumicidins [23]. Despite the high degree of sequence conservation among the rumicidins, the greatest variability was found in the N-terminal region, which also dramatically differed from the N-termini of the previously described PrAMPs. The N-terminal region of rumicidins consists of 10 amino acids, exceeding the N-termini of the peptides from other mammals by at least 5 amino acids (Fig. 3). Although all rumicidins begin with an arginine residue, unlike the previously characterized mammalian peptides, none of the rumicidin representatives have this residue in the second position, which could indicate a somewhat different mechanism of penetration into bacterial cell. Another key difference between the rumicidins and other class I PR-AMP representatives is the change in the consensus sequence (R/K)XX(R/Y)LPRPR, which is necessary for strong interaction with the NPET [13, 39]: in all representatives of the new class, the key functional site (Arg/Tyr)-Leu is replaced by His-Arg. Several representatives of the rumicidins, demonstrating the greatest differences in the N-terminal sequence, were selected for structural and functional characterization: rumicidin 1 (Rum-1) from the Tibetan antelope chiru *Pantholops hodgsonii*, rumicidin 2 (Rum-2) from the African antelope hirola *Beatragus hunteri*, and rumicidin 3 (Rum-3) from the American pronghorn *Antilocapra americana* [23].

The *in vivo* experiments demonstrated absence of lytic activity of rumicidins against the bacterial membrane and involvement of the porin- and

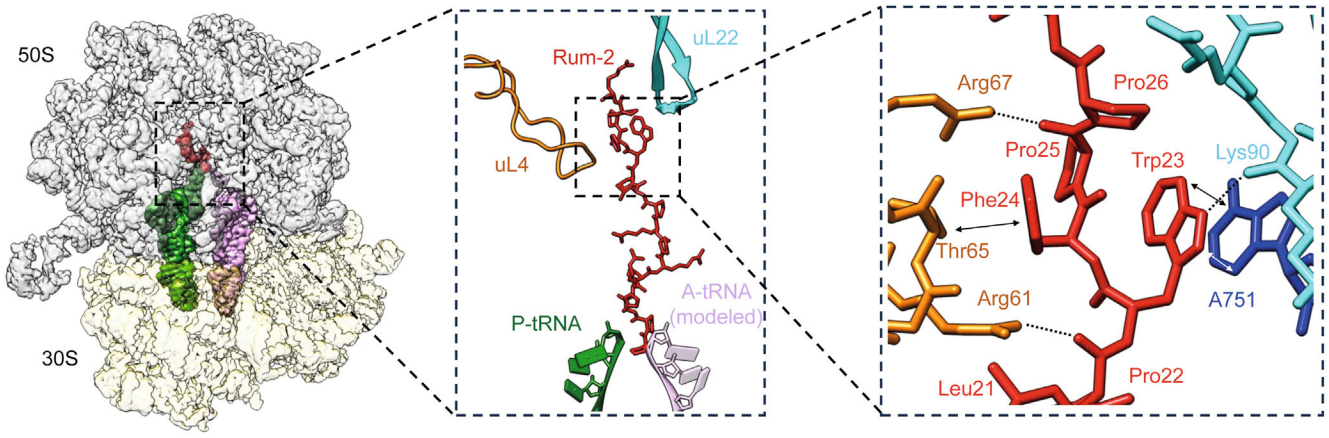

Fig. 4. Interaction of rumicidin 2 (Rum-2) with the bacterial ribosome (PDB ID: 9D89). Rum-2 (shown in red) is located in the NPET of the 70S initiation complex containing the initiator tRNA in the P-site (shown in green), sterically preventing binding of tRNA in the A-site (tRNA model shown in purple). Rum-2 creates a "spacer" at the constriction site of the tunnel, which is formed by the rRNA nucleotides and loops of the ribosomal proteins uL4 (shown in orange) and uL22 (shown in light blue): Pro22 forms a hydrogen bond with Arg61 of the ribosomal protein uL4; Trp23 forms a stacking interaction with the nucleotide A751 of 23S rRNA (shown in blue) and hydrogen bonds with Lys90 of the ribosomal protein uL22; a CH–π-interaction occurs between Phe24 and Thr65 of ribosomal protein uL4.

SbmA-mediated transport for penetration into bacterial cells, while the MacAB-TolC pump was involved in the export of the peptide from the periplasmic space (Fig. 2). The antibacterial effect applies to a number of Gram-negative bacteria belonging to the "ESKAPE" group of pathogens and some mycobacteria. A panel of *in vitro* studies showed that addition of rumicidins led to ribosome stalling at the start codon, prevented formation of the first peptide bond, and, similar to Bac7(1-22), reduced the level of GFP protein fluorescence in the coupled transcription/translation system. Moreover, the structure of one of the class representatives, Rum-2, bound to the 70S initiation complex containing fMet-tRNA^{fMet} in the P-site, showed that rumicidins bind to the NPET in an orientation opposite to the nascent peptide chain (Fig. 4). Thus, it was convincingly shown that rumicidins, despite their differences from the previously described peptides, belong to the class I PrAMPs and inhibit translation by preventing elongation [23].

Quality of the Coulomb potential map detected in the NPET allowed unambiguous fitting of the rumicidin 2 residues 12-27. Density for the first 11 amino acids of the N-terminal part of Rum-2 was not observed, likely due to the high mobility of this region [23]. However, analysis of the sequence of this region of rumicidin suggests some spatial similarity to the previously characterized mammalian peptides [40, 49, 52]. The arginine-rich sequence of the first 6 residues can form a structure resembling the compact N-terminal loop of Bac7 and Tur1A, but this structure is likely somewhat extended into the space of the A-site pocket due to the presence of two prolines at positions 7 and 8, found in all rumicidins.

Truncation of the N-terminus of Bac7 by just 4 amino acids (RRIR) led to the significant (32-fold and more) decrease in antimicrobial activity against *E. coli* and *Salmonella enterica* strains [58]. For the most comparable in length to Bac7(5-35) truncated rumicidins, Rum-1(9-29) and Rum-1(11-29), the MIC values against *E. coli* BW25113 increased only 4-fold (8 μM) compared to the full-length Rum-1 (2 μM) (Table 1). Truncation of the N-terminus of Rum-1 by less than 9 amino acids had virtually no effect (a 2-fold increase in MIC values compared to the full form of the peptide) [23]. Notably, the effect on protein synthesis inhibition in the *in vitro* transcription/translation system differed significantly for the tested variants of Rum-1(9-29) and Rum-1(11-29). Disruption of the reporter protein production was observed at 2 μM concentration of the full-length peptide and 4 μM of Rum-1(9-29), while deletion of two additional amino acid residues increased the concentration required to achieve a similar effect for the truncated peptide (Rum-1(11-29)) by 16-fold (32 μM), indicating a potentially important role of amino acid residues at positions 9 and 10 of rumicidins in inhibiting bacterial ribosomes. Meanwhile, functional significance of the first 8 amino acids of the N-terminus of rumicidins remains unclear, as their deletion did not lead to pronounced disruption of the peptide transport into the cell or significant inhibition of protein biosynthesis. It can be assumed that the unique elongated N-terminal region of rumicidins is important at the stages preceding penetration of the peptide into bacterial cell. Negative effect of N-terminal deletions could also be compensated by the additional features of rumicidins that are absent in other representatives of mammalian PrAMPs.

All rumicidins have two amino acid substitutions in the consensus fragment (R/K)XX(R/Y)LPRPR: the highly conserved (Arg/Tyr)-Leu dyad is replaced by the His-Arg pair, which is not found at these positions in other class I PrAMPs. Meanwhile, the central part of Rum-2 (12-19 amino acids) aligns well with the residues of the consensus sequence of the structurally characterized mammalian peptides and forms similar contacts with rRNA [40, 49, 52] (Fig. 3). Notably, correcting the consensus sequence of rumicidins to the canonical form had no effect on the MIC values and efficiency of the protein synthesis inhibition [23]. While several independent studies have shown that the presence of the Arg-Leu dyad in the Bac7 sequence is crucial for antibacterial activity of the peptide [39, 59].

A unique feature of the rumicidin 2 structure was a clear density of the peptide in the upper and the beginning of the middle part of the NPET, allowing identification of the contacts of deeply located amino acid residues in the NPET [23] (Fig. 4). The most interesting are the contacts between the peptide and constriction area of the NPET, which is formed not only by rRNA nucleotides but also by the loops of ribosomal proteins uL4 and uL22. Pro22 forms a hydrogen bond with Arg61 of the ribosomal protein uL4, Trp23 forms a stacking interaction with the nucleotide A751 of helix H34 of 23S rRNA and hydrogen bonds with Lys90 of the ribosomal protein uL22. A CH–π-interaction occurs between Phe24 and Thr65 of the ribosomal protein uL4. Thus, Rum-2 creates a “spacer” at the constriction site of the NPET [23]. Previously, a similar structure was shown at the constriction site of the NPET for the class II PrAMP Api137: Tyr7 formed an interaction with A751 of the helix H34 of 23S rRNA, and Arg10 formed a hydrogen bond with Arg61 of the ribosomal protein uL4 [53].

It is important to note that for the truncated variant of rumicidin Rum-1(1-22), concentration leading to inhibition of the *in vitro* transcription/translation was 32 μM (16 times higher than for Rum-1), and the MIC against *E. coli* BW25113 was 16 μM (8 times higher than for Rum-1). Meanwhile, the C-terminal truncated form of Bac7, Bac7(1-16), in the similar experiment demonstrated high inhibition efficiency at concentration of 5 μM, and the MIC against *E. coli* BW25113 was 8 μM (2.7 times higher than for Bac7(1-35)) [39]. The MIC values for Bac7(1-16) against *E. coli* and *S. enterica* strains increased only 2-4 times compared to the full-length form [58]. Thus, it can be concluded that for maintaining activity of rumicidins both *in vitro* and *in vivo*, the C-terminal part and the N-terminal part starting from the 9th amino acid (in the case of Rum-1) are necessary, while activity of Bac7 is ensured by the presence of the full N-terminal part.

Importance of the C-terminal part of rumicidins is determined by the presence of a pair of conserved amino acids Trp23 and Phe24 in all representatives of this family, as demonstrated by the N-terminal truncated peptide variant with modifications at these positions. Concentration of Rum-1(9-29, W23A, F24A) required to inhibit protein synthesis in the *in vitro* system was 16 times higher than that of Rum-1 and 8 times higher than of Rum-1(9-29). It is interesting to note that aromatic amino acid residues at the C-terminus are present in many PrAMPs: in the insect peptides (Onc, Pyr), it is tyrosine; in the mammalian peptides, it is one (Bac7, Tur1A) or two (RP-39) phenylalanines; moreover, the Trp-Phe pair was found in the cetacean PrAMP, Lip1 [13]. However, functional role of the Trp-Phe dyad in Lip1, as well as other C-terminal aromatic amino acid residues, has not yet been determined, as most studies have focused on substitutions of amino acid residues in the N-terminal and central parts of the peptides [39, 41]. In this regard, the results of deep mutational scanning studies are extremely interesting, as they revealed that the C-terminal part of Bac7 has great potential for optimization. The study showed that insertion of a tyrosine residue is one of the most significant approaches for increasing activity of the peptide, especially at positions 18 and 19 [59]. Given the presence of a phenylalanine residue at position 20 of Bac7, the appearance of tyrosine in the preceding positions may lead to formation of an aromatic “spacer” that forms specific contacts with amino acid residues and ribonucleotides on the surface of the NPET in the constriction area, similar to rumicidins.

Thus, representatives of the new rumicidin family have an extremely long N-terminal part, differences in the consensus region, and presence of an aromatic “spacer” in the C-terminal region. It can be assumed that fixation of the peptide in the constriction area of the NPET increases the strength of its interaction with the ribosome and can compensate for the partial deletion of N-terminus, which, in the absence of key interaction in the constriction area of the NPET, dramatically reduces antimicrobial effect of the peptide.

Non-canonical positioning of Bac5 in the ribosomal tunnel. Another unusual representative of PrAMPs is Bac5. This peptide (43 amino acids long) was isolated from the bovine neutrophils and consists of an arginine-rich N-terminal region followed by Arg-Pro-Pro-Ile/Phe repeats [26]. For manifestation of antibacterial effect of the peptide, it was crucial to preserve not only the length of the N-terminal region, as truncation of the N-terminus of Bac5 by 4 amino acids led to the loss of its inhibitory properties, but also presence of arginine at the N-terminus or in its immediate vicinity [61]. The variants of Bac5 with deletion of the C-terminal region retained antimicrobial

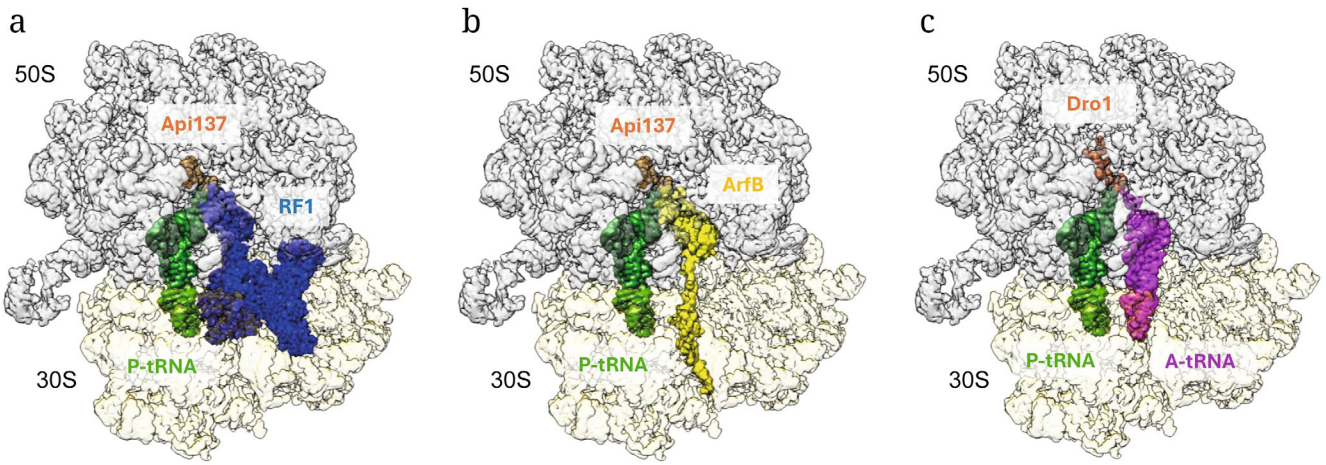

Fig. 5. Interaction of the class II PrAMPs with the termination ribosomal complex (a) (PDB ID: 8AKN, 5O2R), ribosome release complex (b) (PDB ID: 6YSU), and elongation ribosomal complex (c) (PDB ID: 8AM9). P-site tRNA (P-tRNA) is shown in green; release factor RF1 is shown in blue; ribosome-rescue factor ArfB is shown in yellow; A-site tRNA (A-tRNA) is shown in purple; PrAMPs Api137 and Dro1 are shown in orange.

activity, which is a consequence of translation inhibition [16, 62, 63]. Unique sequence of the peptide, consisting of amino acid repeats, shows complete absence of the consensus sequence and even of the previously considered invariant PRP motif. Structural studies of Bac5(1-17) in complex with a vacant bacterial ribosome determined that the peptide adopts an orientation opposite to that of the nascent polypeptide chain. The conformation of Bac5(1-17) in the NPET differs from that of class I (Bac7, Onc112, metalnikowin, Pyr, Tur1A) and class II (Api, drosocin) PrAMPs [16]. Positioning of the N-terminus of the peptide in the PTC suggests potential overlap not only with the CCA-end of the A-site tRNA, as previously demonstrated for all class I peptides, but also with the nucleotide A76 of the initiator $tRNA^{fMet}$. This suggests that Bac5 may have a different mechanism of action and, in addition to disrupting accommodation of the A-site tRNA, could interfere with the correct placement of the initiator tRNA in the P-site of the PTC [16].

INTERACTION OF THE CLASS II PrAMPs WITH THE RIBOSOME

Class II PrAMPs bind within the NPET in an orientation corresponding to that of the synthesized polypeptide chain: their C-terminus extends toward the A-site, while their N-terminus is oriented toward the tunnel exit [48, 53]. Apidaecin-like peptides associate with the ribosome immediately after hydrolysis of the ester bond between the peptide and tRNA and the release of the newly synthesized polypeptide chain [48, 53]. Once bound within the NPET, class II PrAMPs obstruct the dissociation of release factors RF1 and RF2. This stabilization of the termination complex effectively halts the translation cycle by preventing ribosome recycling [48, 53, 64] (Fig. 5). While the overall stability of Api137, a synthetic derivative of natural apidaecin 1b, is mediated by its interaction with the NPET surface, the specific placement of its C-terminal residues Arg17 and Leu18 into the A-site cleft is crucial for translation inhibition [53, 65]. The side chain of Arg17 is coordinated between the residues U2506, G2505, and C2452 of 23S rRNA and Gln235 of the GGQ motif of RF1. Simultaneously, Leu18 residue of the peptide establishes interaction with A76 of the deacylated P-site tRNA [53]. The Api137 peptide containing mutation at position 17, and the truncated Api137(1-16) had significantly lower inhibitory activity compared to the values obtained for the original peptide sequence, emphasizing the key role of the C-terminal Arg17 [45]. Contacts in the constriction site of the NPET are also important: Tyr7 forms a stacking interaction with A751, and Arg10 forms a hydrogen bond with Arg61 of the protein uL4 [53]. This binding mode bears a notable resemblance to the engagement of the aromatic Phe23-Trp24 dyad of rumicidin 2 with the NPET [23]. Additionally, screening of spontaneous *E. coli* mutants resistant to Api137 showed that mutations in the proteins uL22, uL4, and substitutions of the 23S rRNA nucleotides A2059, A2503 increase resistance to Api137 [53].

Drosocin arrests translating ribosomes at the stop codon by forming a water-mediated interaction between its Arg18 residue and Gln235 of the GGQ motif of RF1 [48, 66], which resembles interaction between Arg17 of Api137 and Gln235 of RF1 [53]. Notably, in drosocin, the functionally critical amino acid residues are distributed along the entire sequence, with some located in the N-terminal part. Any substitution of its key arginine residues (Arg9, Arg15, and Arg18),

as well as Lys2 and Pro16, results in a significant decrease of antibacterial activity [48]. Drosocin's high potency is also dependent on a unique modification: O-glycosylation at Thr11 [30, 67, 68], which establishes multiple interactions with rRNA [48].

The class II PrAMPs can retain not only release factors RF1/RF2 but also the ribosome-rescue factor ArfB on the ribosome [69] (Fig. 5). The N-terminal domain of ArfB contains a conserved GGQ motif necessary for catalyzing hydrolysis of peptidyl-tRNA in the P-site [70], and *in vitro* tests confirmed that Api137 traps ArfB after a single peptide hydrolysis round, similar to what occurs with RF1/RF2 [69]. Structural data of an ArfB-ribosome complex with Api137 in the NPET and deacylated tRNA in the P-site confirm that, as in the case of RF1/RF2, Api137 mimics the nascent polypeptide chain. C-terminal Leu18 of Api137 interacts with the ribose of A76 of the P-site tRNA, and the guanidine group of Arg17 interacts with the carbonyl group of the side chain of Gln28 in ArfB's GGQ motif, thereby stabilizing both the tRNA and ArfB [69].

Thus, apidaecin-like peptides disrupt protein synthesis by imposing complex inhibition at the termination stage. Their direct action stalls a small fraction of ribosomes bound to release factors RF1/RF2. However, because the number of ribosomes in an *E. coli* bacterial cell far exceeds the number of available release factors [71], this stall at stop codons has a profound domino effect. It leads to the sequestration of the limited RF pool, causing ribosomes queuing behind the stalled complexes on the mRNA [53, 72]. The release factors pool depletion has two outcomes for the cell: ribosomes that reach a stop codon either stall in the pre-hydrolysis state, or they misread the stop codon by incorporating a closely related aminoacyl-tRNA. The latter results in the expression of the C-terminally extended aberrant proteins [53]. By allowing translation to continue to the 3′-end of mRNA transcripts, the apidaecin-like peptides activate the ribosome rescue system. However, the rescue system is rendered ineffective due to a combination of the depleted termination factors pool, highly efficient blocking of the stop codon of the *arfA* gene mRNA encoding the release factor ArfA, as well as direct capture of the RF-independent ribosome-rescue factor ArfB on the ribosome [69, 72].

Unexpectedly, structural studies of the drosocin-bound ribosomal complexes revealed a small population of 70S ribosomes containing both a deacylated initiator tRNA in the P-site and a dipeptidyl-tRNA in the A-site [66] (Fig. 5). This finding suggests that the class II PrAMPs may also play a role in inhibiting the very first step of elongation. Although the density for the dipeptide moiety of fMet-Leu-tRNA^{Leu} was poorly resolved, the overall configuration indicated a shift of the dipeptide toward Arg18 due to the steric clash of fMet and Val19 residue of drosocin. This configuration could impair the first round of translocation and prevent ribosome progression along the mRNA [66]. This proposed effect of drosocin on early elongation, is supported by *in vivo* data. Ribosome profiling and proteomic analysis demonstrated that Api137 contributed to the ribosome stalling at the start codon of some genes [72]. Among the most affected were genes like *zntA*, *srmB*, *cysS*, *tyrS*, and *yhbL*. Notably, the second codon in each of these mRNAs encodes polar uncharged serine and threonine and hydrophobic leucine, alanine, and isoleucine, respectively [72]. These amino acids are similar in size and structure, and Api137 may specifically stabilize complexes with fMet-Ser/Thr/Leu/Ala/Ile-tRNA in the A-site, mirroring the interaction observed between drosocin and the ribosome in the presence of fMet-Leu dipeptide.

Additional binding sites of apidaecins on the ribosome. Recent structural studies have revealed that synthetic apidaecin 1b derivatives, Api137 and Api88 [73], have two additional binding sites on the 50S ribosomal subunit: near the NPET exit pore (both peptides) and inside domain III of 23S rRNA (only Api88) [25] (Fig. 6). In the first case, the N-terminal part of the peptides is located close to the ribosomal proteins uL29 and uL23 and is attached to the solvent-exposed nucleotides of 23S rRNA A1321, A507, A508, A63, A92, and A93, while their C-terminus is oriented into the NPET. In the second additional site of apidaecin Api88 inside the domain III of 23S rRNA, the N-terminus of the peptide is oriented toward the NPET, and C-terminus points toward the 50S subunit's core. The central Pro9-Arg10-Pro11-Arg12 motif of Api88 is nestled between the helices H51 and H49 of 23S rRNA, ensuring stable positioning of the peptide [25]. The role of Api88's binding inside domain III of 23S rRNA remains unclear, while location of Api137 and Api88 near the exit pore may be crucial for efficient penetration of apidaecin into the NPET. To perform its function, apidaecin must bind to the upper part of the NPET immediately after release of the synthesized peptide but before dissociation of the release factors RF1/RF2 or the ribosome-rescue factor ArfB from the ribosome. Prepositioning near the tunnel entrance likely increases the probability of this molecular event. However, binding of apidaecins in the vestibule of the NPET may have a broader, more strategic function. The NPET exit is formed by ribosomal proteins uL29 and uL23, which serve as a primary binding platform for the trigger factor – a ribosome-associated molecular chaperone involved in the early stages of folding of newly synthesized peptides [74, 75], and the signal recognition particle, which directs membrane proteins for insertion into the plasma membrane [76, 77]. By occupying this region, PrAMPs, could form contacts with these essential

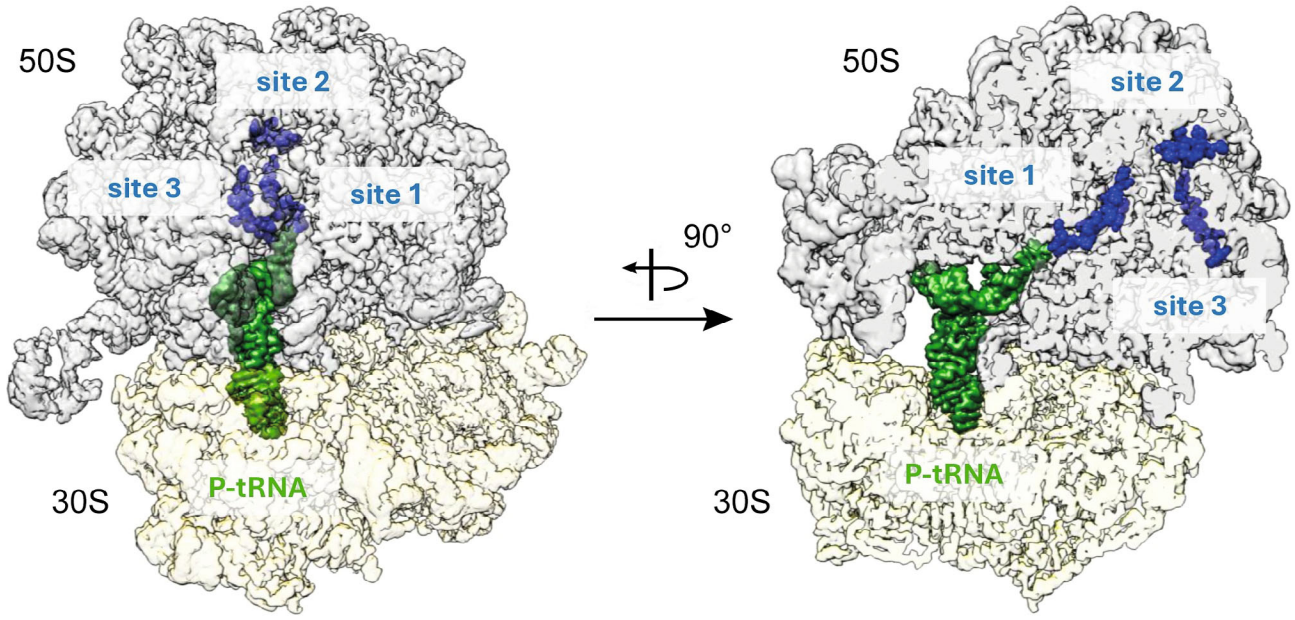

Fig. 6. Binding sites of apidaecins (using Api88 as an example) with the bacterial ribosome (PDB ID: 8RPZ, 8RQ0, 8RQ2). Site 1 – canonical binding site of PrAMPs inside the nascent polypeptide exit tunnel. Site 2 – area near the exit pore of the nascent polypeptide exit tunnel, N-terminal part of Api88 is located near the ribosomal proteins uL29 and uL23, the C-terminus is oriented into the tunnel. Site 3 is located inside domain III of 23S rRNA, the central region of Api88 is located between the helices H51 and H49 of 23S rRNA, and the C-terminus points toward the 50S subunit's core. P-site tRNA (P-tRNA) is shown in green; Api88 is shown in blue.

factors or alter their interaction with the ribosome, disrupting fine regulation of the functional protein formation after biosynthesis [25].

Beyond their action on mature 70S ribosomes and 50S ribosomal subunits, apidaecins interact with precursors of 50S subunits, disrupting the ribosome particle assembly process within the cell [78]. Treatment of bacterial cells with Api137 leads to the appearance of an additional peak on the ribosomal profile, which was attributed to the pre-50S state. This effect was not observed with the structurally similar Api88 or the class I PrAMP Onc112 [79]. Structural studies reveal that Api137 leads to the appearance of four distinct pre-50S intermediates, differing in structural completeness both in terms of proteins and structural regions of 23S rRNA [78]. These intermediates are largely assembled but share a critical defect: the functional core, including the PTC, located inside the central protuberance of domain V, and adjacent rRNA elements, remain unstructured. In many precursors, proteins uL22 and uL29 were absent, which led to the change in the 50S assembly pathway. Notably, Api137 does not bind within the NPET of these premature subunits, as apparently, the tunnel must be fully formed for peptide binding. However, Api137 was found in the additional site [25], occupying a position near the exit pore of the NPET in close proximity to the sites of uL22 and uL29, creating steric hindrance for the inclusion of these proteins [78].

STRENGTH OF PrAMP INTERACTION WITH THE RIBOSOME

Class II PrAMPs interact with diverse ribosomal complexes, forming contacts only with the NPET surface at the initial stages of elongation [66, 72] or additionally with the ligands (P-site tRNA, RF1, ArfB) during termination or ribosome rescue process [53, 66, 72]. The formation of complexes with different peptide-binding modes was confirmed experimentally using both ribosomal extracts containing additional protein factors and purified ribosome preparations. The dissociation constants (K_d) values obtained using the fluorescence polarization method [80] using 5(6)-carboxyfluorescein-labeled Api137 and ribosomal extract ranged from 0.34 to 0.62 μM [45, 81, 82], while those determined using purified 50S and 70S preparations were 2.2 ± 0.1 μM and 4.7 ± 0.3 μM, respectively (Table 2) [25]. The K_d values for Api88 were comparable in the ribosomal extract (0.7-1.31 μM) [45, 81, 82] and in the purified 50S and 70S preparations (0.58 ± 0.05 μM and 1.82 ± 0.08 μM, respectively) [25]. The obtained values are consistent with the described models of apidaecin-ribosome interactions [25, 53, 72]. Api137 is stabilized within the NPET by additional factors, which explains its order-of-magnitude stronger binding to ribosomes in the extract compared to isolated 70S ribosomes. In contrast to Api137, Api88 has a weak effect on termination [25]. It appears

that additional factors do not contribute significantly to its stabilization on the ribosome, so its K_d values in the extract and for purified 70S ribosomes are similar and higher than those measured for Api137. Notably, the additional binding sites for Api137 and Api88 have been identified on the 50S subunit [25], and therefore, the observed K_d values represent a superposition of affinities for each individual site. The authors suggest the potential existence of an even greater number of sites, which may differ between the 50S and 70S particles. This may explain the 2-3-fold differences in K_d values observed between 50S and 70S ribosome [25]. Further studies aimed at characterizing the interactions of these peptides with the trigger factor and signal recognition particle, as well as investigating how PrAMPs binding alters the affinity of these protein complexes to the ribosome and impairs their function, will be crucial to determine whether binding to additional sites is essential for their antibacterial activity. For such investigations, 50S subunits or 70S ribosomes lacking proteins uL22 and uL29, which appear to be necessary for PrAMPs binding at the canonical site, could be employed [25].

In contrast to the apidaecin-like peptides, all class I PrAMPs share a conserved binding mode, positioning their N-terminus within the A-site pocket of the upper part of NPET. The remarkable consistency of K_d values for Onc112 determined using ribosomal extract (0.023-0.093 μM) [45, 81, 82] and purified 50S and 70S particles (0.050 ± 0.001 μM and 0.060 ± 0.005 μM, respectively) [25], is likely due to the property of Onc112 to bind to the ribosome without involvement of additional stabilizing factors. This high affinity of Onc112, which is comparable to that of conventional NPET-binding antibiotics (for example, streptogramins B antibiotics quinupristin and linopristin bind to the 70S ribosome with a K_d of 0.04 ± 0.02 μM and 0.040 ± 0.006 μM, respectively [83]), likely results from extensive interactions within the tunnel. However, deletion of two N-terminal residues of oncocin led to disruption of the peptide binding to the ribosome [49], indicating predominant importance of the N-terminal region of the peptide. Structural studies of mammalian peptides also revealed formation of a compact, positively charged loop anchoring PrAMPs in the A-site pocket region [40, 52]. Such non-specific interaction could lead to the significant stabilization of the peptide in the NPET due to formation of an occluded state and could be more important than the contacts within the tunnel. Along with the N-terminal “anchor,” the aromatic “spacer” of rumicidins, forming specific contacts with amino acid residues of the proteins uL4 and uL22 and ribonucleotides on the surface of the NPET in the constriction area [23], could be a key element ensuring even stronger interaction of some class I PrAMPs members with ribosomal complexes. However, determining contribution of these peptide elements requires further research.

Table 2. K_d values of PrAMPs. Preparations labeled as 30S, 50S, 70S are purified ribosomal subunits and ribosomes

Peptide	K_d, μM	Preparation	References
Onc112	0.023-0.093	70S extract	[45, 81, 82]
	0.060 ± 0.005	70S	[25]
	0.050 ± 0.001	50S	[25]
	1.14 ± 0.06	30S	[25]
Api137	0.34-0.62	70S extract	[45, 81, 82]
	4.7 ± 0.3	70S	[25]
	2.2 ± 0.1	50S	[25]
	20 ± 8	30S	[25]
Api88	0.7-1.31	70S extract	[45, 82]
	1.82 ± 0.08	70S	[25]
	0.58 ± 0.05	50S	[25]
	14 ± 3	30S	[25]

CONCLUSION AND PERSPECTIVES

PrAMPs open up great perspectives as an alternative to traditional antibiotics. Their mechanism of action based on disruption of bacterial translation – one of the fundamental processes of life – emphasizes high therapeutic potential of these compounds. The main advantages of PrAMPs are their broad spectrum of antimicrobial action, low probability of resistance development, and minimal toxicity to animal and human cells. However, there are several obstacles to the clinical use of PrAMPs. These include rapid peptide degradation *in vivo*, low bioavailability upon oral administration, complexity and high cost of industrial synthesis, as well as insufficient clinical data confirming their safety. At the current stage of PrAMPs introduction into clinical practice, targeted optimization of the peptide structures is necessary to enhance their stability and boost antimicrobial activity. Along with mutational screening, expanding the “library” of PrAMPs by discovering new members of this class and conducting studies of the poorly investigated peptides, appear promising. Work in this direction has led to the discovery of a unique feature of rumicidins – formation of a strong interaction

with the ribosome due to location of the conserved Trp23-Phe24 dyad in the constriction site of the exit tunnel. This finding could serve as the foundation for designing peptides that surpass natural ones in antibacterial properties.

Thus, PrAMPs represent one of the most promising avenues in the development of new antibacterial agents. Their further study and optimization offer extensive opportunities for transforming approaches to treating infectious diseases.

Abbreviations

AMP	antimicrobial peptide
MIC	minimum inhibitory concentration
NPET	nascent polypeptide exit tunnel
PrAMP	proline-rich antimicrobial peptide
PTC	peptidyltransferase center
Api	apidaecin
Bac	bactenecin
Onc	oncocin
Pyr	pyrrhocoricin
Rum	rumicidin

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Ethics approval and consent to participate

This work does not contain any studies involving humans and animal subjects performed by any of the authors.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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