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Short communication

Activities of four frog skin-derived antimicrobial peptides (temporin-1DRa, temporin-1Va and the melittin-related peptides AR-23 and RV-23) against anaerobic bacteria

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Abstract

The activities of two antimicrobial peptides belonging to the temporin family (temporin-1DRa from *Rana draytonii* and temporin-1Va from *Rana virgatipes*) and two peptides with structural similarity to the bee venom peptide melittin (AR-23 from *Rana tagoi* and RV-23 from *R. draytonii*) were evaluated against a range of reference strains and clinical isolates of anaerobic bacteria. These peptides were selected because they show broad-spectrum growth inhibitory activity against reference strains of several medically important aerobic microorganisms and against clinical isolates of methicillin-resistant *Staphylococcus aureus*. All peptides showed relatively high potency (minimum inhibitory concentration (MIC) $\leq$25 μM) against the Gram-positive bacilli *Propionibacterium acnes* and *Clostridium tertium* and the Gram-positive cocci *Peptostreptococcus anaerobius.* Activity was lower and more variable against *Clostridium septicum, Clostridium perfringens* and *Peptostreptococcus asaccharolyticus*. Growth of the Gram-negative bacilli *Bacteroides fragilis* and *Fusobacterium* spp. was poorly inhibited, but all the peptides were active (MIC $\leq$ 25 μM) against *Prevotella melaninogenica*. The clinical utility of the melittin-related peptides is limited by their toxicities, but temporin-1DRa and temporin-1Va have relatively low haemolytic activity against human erythrocytes and so represent candidates for drug development, particularly for topical therapy of infected surface lesions.

Keywords: Anaerobic bacteria; Antimicrobial peptide; Temporin; Melittin; Frog skin

1. Introduction

Cationic peptides with antibacterial and antifungal activities are widely distributed in nature and constitute a component of the innate immune system, which is the first line of defence against invasion by microbial pathogens both for vertebrates and invertebrates. These antimicrobial peptides generally do not contain domains of conserved amino acid residues but may be divided into three main classes on the basis of secondary structure: (a) peptides that form an amphipathic α-helix in a membrane-mimetic environment; (b) peptides containing one or more pairs of cysteine residues that form a β-sheet structure; and (c) glycine and/or proline-rich peptides [1]. The emergence, particularly during the past 10–15 years, of strains of pathogenic bacteria and fungi that are resistant to commonly used antibiotics has necessitated a search for new types of antimicrobial agent, and anti-infective drugs based upon these naturally occurring peptides are receiving increasing attention. Although development of resistance to peptide-based drugs has been demonstrated experimentally, it is believed to take place at much lower rates compared with conventional antibiotics. On the negative side, however, their cytolytic activities against

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mammalian cells and their short half-lives in the circulation mean that future therapeutic applications are more likely to involve topical rather than systemic administration.

Frogs of the genus *Rana* (Ranidae), a group of ca. 250 extant species distributed worldwide, have proved to be a particularly rich source of peptides with antimicrobial activities [2]. These peptides are synthesised in granular glands in the skin and are released into skin secretions, often in very high concentration, in response to infection or environmental stress. More than 100 different compounds have been purified and characterised. The *Rana* skin peptides are almost invariably cationic, relatively hydrophobic and have the propensity to form an amphipathic α-helix [1]. On the basis of limited structural similarity, they may be divided into families that are believed to be related evolutionarily, having arisen from a series of gene duplication events [2].

Despite the fact that anaerobic pathogens are responsible for a wide range of human infections and that antibiotic resistance among them is increasing [3], there have been relatively few investigations of the actions of cationic antimicrobial peptides on anaerobic bacteria [4–6]. This is, at least in part, a consequence of the technical difficulties associated with isolation and culture of anaerobic species. The present study investigates the growth inhibitory activity of synthetic replicates of four peptides, first isolated from the skins of ranid frogs, against a range of reference strains and clinical isolates of Gram-positive and Gram-negative anaerobes. The peptides selected for this study (temporin-1DRa from *Rana draytonii* [7], temporin-1Va from *Rana virgatipes* [8], AR-23 from *Rana tagoi* [9] and RV-23 from *R. draytonii* [7]), show broad-spectrum growth inhibitory activity against aerobic bacteria and against the opportunistic yeast pathogen *Candida albicans*. Peptides AR-23 and RV-23 show structural similarity to the bee venom peptide, melittin, and so their antimicrobial properties are compared with those of this well-characterised cytolytic peptide.

2. Materials and methods

2.1. Bacteria

Reference strains of aerobic bacteria were purchased from the American Type Culture Collection (ATCC, Rockville, MD), from the National Collection of Type Cultures (London, UK) and from the Hungarian National Type Culture Collection (Budapest, Hungary). The properties of the clinical isolates of multidrug-resistant *Staphylococcus aureus* have been described previously [10]. Anaerobic strains isolated from six different clinical specimens and six reference strains (ATCC) belonging to different species were used in this study. The clinical strains were identified by conventional methods and ATB raid ID 32A (bioMérieux, Marcy l'Etoile, France). The strains were grown on pre-reduced anaerobic blood agar plates (Columbia agar base (Oxoid, Basingstoke, UK) supplemented with 5% cattle blood, 1 mg/L vitamin K_1 and haemin) and incubated at 37 °C for 48 h under anaerobic conditions (85% N_2, 10% CO_2, 5% H_2) in a Concept 400 anaerobic chamber (Biotrace Inc., Cincinnati, OH). Isolates of *Clostridium septicum* 1176, *Prevotella melaninogenica* 26117 and *Peptostreptococcus anaerobius* 11422 were recovered from infected foot ulcers of diabetic patients. Strains of *Bacteroides fragilis* R19811 and *Propionibacterium acnes* 669 were isolated from blood cultures, and *Fusobacterium nucleatum* 33894 was isolated from a skin infection.

2.2. Peptides

Peptide AR-23 (AIGSILGALAKGLPTLISWIKNR.NH_2), peptide RV-23 (RIGVLLARLPKLFSLFKLMGKKV), temporin-1DRa (HFLGTLVNLAKKIL.NH_2) and temporin-1Va (FLSSIGKILGNLL.NH_2) were supplied in crude form by GL Biochem (Shanghai) Ltd. (Shanghai, China) and purified to near homogeneity by reverse-phase high performance liquid chromatography on a 2.2 cm × 25 cm Vydac 218TP1022 (C-18) column (Separations Group, Hesperia, CA) equilibrated with acetonitrile/water/trifluoroacetic acid (21.0/78.9/0.1) at a flow rate of 6 mL/min. The concentration of acetonitrile was raised to 56% over 60 min using a linear gradient. The final purity of the synthetic peptides was >95% and their identities were confirmed by electrospray mass spectrometry. Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) was supplied by Sigma Chemical Co. (St Louis, MO) and was used without further purification.

2.3. Antimicrobial assays

Minimum inhibitory concentrations (MICs) of the peptides against aerobic microorganisms were determined in duplicate in two independent experiments by standard microdilution methods using 96-well microtitre cell culture plates as previously described [10].

MICs of the peptides against anaerobic bacteria were determined by a standard microdilution method [11]. The inoculum was prepared in *Brucella* broth (pH 7.2) to achieve 10^6 colony-forming units/mL. Serial dilutions of the peptides in 50 μL *Brucella* broth (pH 7.2) were mixed with an inoculum (50 μL) of the different strains and the microtitre plates were incubated for 48 h at 37 °C in an anaerobic chamber before reading the MIC values.

2.4. Haemolysis assay

The haemolytic activities of the peptides against human erythrocytes was determined as described previously [10]. The LD_{50} value was taken as the mean concentration of peptide producing 50% haemolysis in three independent experiments.

Table 1
Minimum inhibitory concentrations (MICs) against reference strains of aerobic microorganisms and haemolytic activity against human erythrocytes of synthetic antimicrobial peptides

Strain	MIC (μM)				
	Melittin	AR-23	RV-23	Temporin-1DRa	Temporin-1Va
Escherichia coli ATCC 25922	6	12.5	6	12.5	25
Klebsiella pneumoniae KK3 9904	12.5	50	12.5	25	50
Enterobacter cloacae HNTCC 53001	12.5	12.5	6	25	50
Pseudomonas aeruginosa ATCC 27853	12.5	25	6	25	100
Salmonella typhimurium LT2	25	50	25	25	50
Proteus mirabilis ATCC 25933	>100	>100	>100	>100	>100
Staphylococcus aureus NCTC 8325	3	6	25	6	12.5
Staphylococcus epidermidis RP62A	3	3	6	6	25
Enterococcus faecalis ATCC 29212	6	6	12.5	25	50
Streptococcus group B HNTCC 80130	6	12.5	12.5	12.5	25
Candida albicans ATCC 90028	6	12.5	50	25	100
LD_{50} erythrocytes (μM)	0.6	8	35	70	120

LD_{50}, mean concentration of peptide producing 50% haemolysis in three independent experiments.

3. Results

The MIC values (μM) of synthetic replicates of the four frog skin peptides and the bee venom peptide melittin against reference strains of aerobic microorganisms and the LD_{50} values (μM) for lysis of human erythrocytes are shown in Table 1. The MIC values of the peptides against clinical isolates of methicillin-resistant *S. aureus* (MRSA) with previously characterised resistance to antibiotics [10] are shown in Table 2. The MIC values of the peptides against reference strains and clinical isolates of anaerobic bacteria are shown in Table 3.

All peptides were active (MIC ≤ 50 μM) against the strains of Gram-negative and Gram-positive aerobic bacteria tested, with the exception of *Proteus mirabilis*, and were also active against *C. albicans* (Table 1). The peptides inhibited the growth of multidrug-resistant strains of MRSA (Table 2). Melittin and the structurally related peptide AR-23 were the most potent but also the most toxic as measured by haemolysis of human erythrocytes. The effects of the frog skin peptides and melittin on anaerobic bacteria were more variable (Table 3). All peptides showed relatively high potency (MIC ≤ 25 μM) against a reference strain and a clinical isolate of the Gram-positive bacillus *P. acnes* and were active (MIC ≤ 50 μM) against the Gram-positive cocci *Peptostreptococcus asaccharolyticus* and *P. anaerobius* (Table 3). Among the Gram-positive spore-forming bacilli, all peptides showed high potency (MIC ≤ 12.5 μM) against *Clostridium tertium* but activities against *C. septicum* and *Clostridium perfringens* were more variable. For example, AR-23 was inactive against *C. perfringens* whereas the structurally related peptide RV-23 showed quite high potency (MIC = 12.5 μM). With the exception of *P. melaninogenica,* which was susceptible (MIC ≤ 25 μM) to all peptides tested, activity of the frog skin peptides against Gram-negative bacilli was appreciably less than against the Gram-positive anaerobes. Temporin-1DRa was the only peptide to show activity (MIC = 50 μM) against a reference strain of *B. fragilis* and all were inactive against a multidrug-resistant clinical isolate. Similarly, with the exception of temporin-1DRa against *F. nucleatum* (MIC = 25 μM), potencies against *Fusobacterium* spp. were low.

Table 2
Minimum inhibitory concentrations (MICs) of synthetic antimicrobial peptides against clinical isolates of methicillin-resistant *Staphylococcus aureus*

Strain	MIC (μM)				
	Melittin	AR-23	RV-23	Temporin-1DRa	Temporin-1Va
T7/5	2.5	2.5	20	10	20
T7/20	2.5	2.5	20	10	20
T17/13	2.5	2.5	10	10	20
T27/9	5	5	20	10	40
T58/7	20	20	20	10	40
V4180	2.5	2.5	10	10	40

4. Discussion

The aim of the present study was to investigate the in vitro efficacy of four peptides, initially isolated from the skin of ranid frogs, against a range of anaerobic bacteria associated with human infections. The peptides were selected because of their comparative ease of chemical synthesis, high solubility in aqueous media and relatively high potency against aerobic bacteria. Peptides of the temporin family are widely distributed among species of Eurasian and New World ranids and more than 50 different members of the family have been identified to date [2]. Most temporins show growth inhibitory activity only against Gram-positive bacteria but, as shown in Table 1, temporin-1DRa and temporin-1Va are atypical in displaying activity against reference strains of several clinically relevant Gram-negative species and against the opportunistic yeast pathogen *C. albicans*. Peptides AR-23 and RV-23 show limited structural similarity to the bee venom peptide melit-

Table 3
Minimum inhibitory concentrations of synthetic antimicrobial peptides against reference strains and clinical isolates of anaerobic bacteria

Strain	MIC (μM)				
	Melittin	AR-23	RV-23	Temporin-1DRa	Temporin-1Va
Bacteroides fragilis ATCC 25285	12.5	>50	>50	50	>50
Bacteroides fragilis R19811	12.5	>50	>50	>50	>50
Prevotella melaninogenica 26117	12.5	3	6	12.5	25
Fusobacterium varium ATCC 27725	50	50	50	>50	50
Fusobacterium nucleatum 33894	>50	>50	>50	25	50
Clostridium tertium ATCC 19465	6	12.5	12.5	3	12.5
Clostridium perfringens ATCC 10543	12.5	>50	12.5	6	25
Clostridium septicum 1176	50	12.5	50	12.5	>50
Propionibacterium acnes ATCC 11828	12.5	12.5	12.5	6	6
Propionibacterium acnes 669	12.5	25	6	6	12.5
Peptostreptococcus asaccharolyticus ATCC 29743	25	50	50	25	50
Peptostreptococcus anaerobius 11422	25	25	25	12.5	25

tin, but the evolutionary relationship between the peptides is unclear. AR-23 and RV-23 also show potent growth inhibitory activity against a range of Gram-positive and Gram-negative aerobic bacteria and against *C. albicans* (Table 1).

The four peptides investigated in this study show potential for development into therapeutically valuable antimicrobial agents with particular applicability to treatment of infected surface lesions such as foot ulcers in diabetic patients. Foot ulcers are among the most common complications of diabetes mellitus and, without therapeutic intervention, these ulcers deteriorate to deep infection or gangrene, resulting in amputation. Antimicrobial therapy for infected diabetic foot ulcers is becoming increasingly challenging for physicians because of the emergence of drug-resistant pathogens and there is an urgent need to develop novel agents to which the microorganisms have not been exposed. Infections are generally polymicrobial and in a comprehensive survey involving 825 patients [12] it was found that Gram-positive aerobes, particularly *S. aureus, Staphylococcus epidermidis, Enterococcus faecalis* and Group B *Streptococcus*, represented the most frequent organisms recovered from the infected ulcers. The most common Gram-negative aerobes recovered were *Pseudomonas aeruginosa*, *Escherichia coli*, *P. mirabilis* and *Acinetobacter* spp. Whilst obligate anaerobes are seldom, if ever, the sole bacterial species isolated from foot ulcers, *Peptostreptococcus* spp., *Prevotella* spp. and *Bacteroides* spp. are often recovered together with aerobes. The four frog skin peptides investigated in the present study show potent growth inhibitory activity against the most frequently recovered aerobic and anaerobic bacteria, with the exception of *P. mirabilis* and *B. fragilis*. The resistance of *P. mirabilis* to a wide range of peptide-based antimicrobial agents arises, at least in part, from the secretion of a metalloprotease that degrades the peptides by proteolytic cleavage. It has been shown that temporin-1Va is rapidly cleaved primarily at positions Leu-2 and Ile-5, and temporin-1DRa at positions Leu-9 and Ile-13 by this enzyme (J.M. Conlon, unpublished data). The reason why *B. fragilis* was susceptible to melittin but not to the other peptides tested is unclear but secretion of a metalloprotease by enterotoxigenic strains of this organism has been documented [13].

The strong haemolytic activity of melittin ($LD_{50} < 1$ μM) and peptide AR-23 ($LD_{50} = 8$ μM) against human erythrocytes severely limits the potential of the peptides for development into a therapeutically valuable anti-infective drug, particularly for systemic use. However, the haemolytic activities of temporin-1DRa ($LD_{50} = 70$ μM) and temporin-1Va ($LD_{50} = 120$ μM) are appreciably less. The cytolytic activities of these peptides against L929 fibroblasts are also more than 10-fold less than that of melittin (J.M. Conlon, unpublished data).

The susceptibility of *P. acnes* to the frog skin peptides raises the possibility of their development into agents for the topical treatment of acne vulgaris, a disease of the pilosebaceous unit with both bacterial and inflammatory components. *Propionibacterium acnes* is found in normal human cutaneous flora and it is thought that colonisation and proliferation by this organism play a major role in the development of an acne lesion. Bacterial colonisation is preceded by hyperproliferation of keratinocytes and increased sebum secretion in a hair follicle. Previous studies have shown that cationic antimicrobial peptides, as well as possessing microbicidal actions, will also reduce the inflammatory response produced by bacteria [14,15]. Thus, further studies are warranted to determine whether the frog skin peptides may exercise a dual beneficial role in acne treatment by manifesting a bactericidal action on *P. acnes* and an anti-inflammatory effect on host cells.

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