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Enhanced antimicrobial activity of novel synthetic peptides derived from vejovine and hadrurin☆

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ABSTRACT

Background: Microbial antibiotic resistance is a challenging medical problem nowadays. Two scorpion peptides displaying antibiotic activity: hadrurin and vejovine were taken as models for the design of novel shorter peptides with similar activity.
Methods: Using the standard Fmoc-based solid phase synthesis technique of Merrifield twelve peptides (18 to 29 amino acids long) were synthesized, purified and assayed against a variety of multi-drug resistant Gram-negative bacteria from clinical isolates. Hemolytic and antiparasitic activities of the peptides and their possible interactions with eukaryotic cells were verified. Release of the fluorophore calcein from liposomes treated with these peptides was measured.
Results: A peptide with sequence GILKTIKSIASKVANTVQKLKRKAKNAVA), and three analogs: Δ(A29), Δ(K12-Q18; N26—A29), and K4N Δ(K12-Q18; N26—A29) were shown to inhibit the growth of Gram-negative (*E. coli* ATCC25922) and Gram-positive bacteria (*S. aureus*), as well as multi-drug resistant (MDR) clinical isolated. The antibacterial and antiparasitic activities were found with peptides at 0.78 to 25 μM and 5 to 25 μM concentration, respectively. These peptides have low cytotoxic and hemolytic activities at concentrations significantly exceeding their minimum inhibitory concentrations (MICs), showing values between 40 and 900 μM for their EC_{50}, compared to the parent peptides vejovine and hadrurin that at the same concentration of their MICs lysed more than 50% of human erythrocytes cells.
Conclusions: These peptides promise to be good candidates to combat infections caused by Gram-negative bacteria from nosocomial infections.
General significance: Our results confirm that well designed synthetic peptides can be an alternative for solving the lack of effective antibiotics to control bacterial infections.

1. Introduction

The phenomenon of antimicrobial resistance is one of the biggest health challenges faced today by the medical community. This problem has created an urgent need for the development of novel classes of antimicrobial agents [1,2]. Unfortunately, the number of new antibiotics in the pipeline of the leading pharmaceutical companies has been declining because they have shifted their attention towards more lucrative areas of drug development [3,4]. A promising alternative for today's antibiotics is antimicrobial peptides (AMPs), since they have shown a broad spectrum of activity against pathogens (bacteria, fungi, parasites and virus) and induce killing in a short contact time [5–7]. They are usually gene-encoded peptides that are expressed either constitutively or are inducible (through signal received from infectious or inflammatory agents). All natural antimicrobial peptides share many common features, including small size (generally 12–50 amino acid residues long), cationic character (with an overall net charge ranging from +2 to +9) and an

Abbreviations: AMPs, antimicrobial peptides; ATCC, American type culture collection; CD, circular dichroism; CFU, colony forming units; CLSI, Clinical and Laboratory Standards Institute; DIPEA, N,N-diisopropylethylamine; DMEM, Dulbecco's modified eagle's medium; DMF, N,N-dimethylformamide; Fmoc, 9-fluorenylmethoxicarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, N-hydroxibenzotriazole hydrate; MIC, minimal inhibitory concentration; MDR, multi-drug resistant; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H- tetrazolium); POPC, L-α-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol); RP-HPLC, reverse-phase high-performance liquid chromatography; SUVs, small unilamellar vesicles; TFA, trifluoroacetic acid

☆ A patent on Novel Hybrid Antibiotic Peptide and its Variants was deposited in Mexico (IMP Folio MX/E/2011/044744).

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amphipatic structure (containing ~50% hydrophobic residues) [8,9]. Despite their sequence diversity, AMPs can be classified into four major classes: (a) α-helical peptides, (b) β-sheet stabilized by two or three disulphide bridges, (c) extended helices with a predominance of one or more amino acids (like histidine, arginine and proline) and (d) loop forming structures [10–12]. At present time, considerable effort has been made to elucidate the mode of action of antimicrobial peptides. However, the molecular mechanism by which they exert activity against microorganisms is not clear [13]. It is generally agreed that AMPs perturb membranes by forming transient pores via one of the various models proposed to account for this effect, i.e. barrel-stave, carpet-like, toroidal (or wormhole) pore formation, detergent-type micellization, and induction of non-lamellar phases, leading to membrane permeabilization and either leakage of cell content and osmotic instability, and/or peptide diffusion to intracellular targets [13–15]. The most abundant and widely studied naturally-occurring types are the linear, amphipathic and α-helical peptides [16]. In a search for such new antibiotics, peptides with antimicrobial activity from the venom of scorpions were found. The first molecule isolated was a defensin from the hemolymph of *Leiurus quinquestriatus Hebraeus* [17]. Subsequently, IsCTs 1 and 2 were obtained from *Opisthacanthus madagascariensis* [18]. Furthermore, opistoporines 1 and 2 were isolated from *Opistophtalmus carinatus* [19]. In addition, scorpine was isolated from the scorpion *Pandinus imperator*, which showed antibacterial and antiparasitic activity, against *Plamodium berghei* [20]. Recently, from *Vejovis mexicanus* and *Hadrurus gertschi*, two novel peptides were identified: vejovine [21] and hadrurin [4]. Although they showed activity against multidrug resistant bacteria they also lyse eukaryotic cells (assayed by measuring hemolytic activity of human erythrocytes). Therefore, one approach to generate highly active antimicrobial peptides, which possess a low hemolytic activity, is to design novel analogs based on the structure of natural peptides, but changing physico-chemical properties that are known to influence the cytolytic activity [8,20]. Among the properties taken into account are: helicity, hydrophobicity, hydrophobic moment, polar angle, net positive charge and total number of amino acids [22]. This strategy was applied here and produced peptides with better microbicide activity and less toxicity towards host cells than vejovine and hadrurin, in view of potential therapeutic applications [5,21]. From twelve newly synthesized peptides following this strategy, four are shown to be effective in inhibiting bacterial growth, including bacteria strains isolated from a hospital clinical setting, and are promising leading compounds for the development of novel antibiotics.

2. Materials and methods

2.1. Material

Amino acids protected by 9-fluorenylmethoxicarbonyl (Fmoc) and Fmoc-peptide amide linker resin were obtained from Novabiochem (La Jolla, CA). Other materials were: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), from ChemPep Inc., (Wellington, FL); N-hydroxibenzotriazole hydrate (HOBt), from LC Sciences (Houston, TX); N,N-diisopropylethylamine (DIPEA) from Sigma-Aldrich (St. Louis, MO); trifluoroacetic acid (TFA), from American Bioanalytical Inc. (Natick, MA); analytical grade N, N-dimethylformamide (DMF) from Aldrich Chemical Co. Inc. (Milwaukee, WI); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H- tetrazolium), abbreviated MTS was from Promega (Madison, WI); DMEM was supplied by HyClone (Logan, UT); 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) sodium salt (POPG) was purchased from Sigma (St. Louis, MO); calcein from Molecular Probes (Grand Island, NY.); L-α-phosphatidylcholine (Egg POPC, Chicken-99%) and cholesterol were obtained from Avanti Polar Lipids Inc. (Alabaster, AL); and fetal bovine serum (FBS) from Gibco (Carisbad, CA). HEK 293 and COS 7 cell lines were purchased from American Type Culture Collection (ATCC; Bethesda, MD). Analytical C_{18} RP-HPLC column (5 mm i.d.×250 mm) was from Vydac (Hysperia, CA). All other reagents were analytical grade products. The buffers were prepared with double quartz-distilled water.

2.2. Bacterial strains

Escherichia coli ATCC25922, *S. pneumonaie* ATCC49619 and *Staphylococcus aureus* ATCC25923 were purchased from the American Type Culture Collection. MDR clinical isolates, which are non-susceptible to at least one agent in three or more antimicrobial categories [23] and causing nosocomial infections, were obtained from the Center for Research on Infectious Diseases collection of the National Institute of Public Health, Cuernavaca, Morelos, Mexico. The various strains include the following isolates: *E. coli* 170 [7], 4530, 5580 and 09240; *E. cloacae* 2524, 6780, 06266 and 06268; *K. pneumoniae* 913, 1625 [24], 068228 and 14218, which are extended-spectrum ß-lactamase-producers, in consequence they are resistant to all penicillins and cephalosporins; *P. aeruginosa* 3599, 4660 [25], 5106 and 6102 which are metallo-ß-lactamase-producers and are resistant to all ß-lactam antibiotics including carbapenems; *A. baumannii* 5821, 5825, 5838, 5852, 7804, 7839 and 7847 (carbapenem resistant); *S. pneumoniae* 150, penicillin resistant; *S. typhimurium* 2205, 2211 and 2217 (cephalosporins resistant).

2.3. Design of AMPs based on structural determinants

AMPs were designed based on three major structural determinants, which include: hydrophobicity (H), hydrophobic moment (μH) and charge (Q), taking care that all peptides used in this work conserved an amphipatic and helicoidal conformation [26]. Twelve synthetic AMPs were designed with hydrophobicity from −0.203 to −0.292, hydrophobic moment of 0.422 to 0.639 and net charges of +5 to +8. The amino acid sequences, molecular mass and structural parameters determined for the 12 AMPs used in this study are summarized in Fig. 1B and Table 1.

2.4. Peptide synthesis and purification

Peptides listed in Fig. 1B were prepared manually using the standard Fmoc-based solid phase synthesis technique (Merrifield, 1963) on Rink amide MBHA resin (0.54 mol/g resin) [11]. HBTU and HOBt were used as coupling reagents, and double fold excess of Fmoc amino acids was added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/phenol/thioanisole/dithiotreitol/H_2O (84:5:5:1:5, v/v) for 2 h at room temperature, crude peptides were repeatedly extracted with diethyl ether and purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a C_{18} analytical column using an appropriate water/acetonitrile gradient in the presence of 0.1% TFA for 60 min (purity >98%). The molecular masses of purified peptides were determined by electrospray ionization mass spectrometry (ESI-MS) using Mass Spectrometer LCQ^{Duo} (Thermo electron/Finningan, San Jose, CA).

2.5. Antibacterial assays

Initially the antibacterial activity of the peptides was qualitatively measured following the Kirby–Bauer method (1996), according to the CLSI (Clinical and Laboratory Standards Institute) recommendations [27]. Briefly, Petri dishes containing Müeller-Hinton agar were sown with bacteria inoculums from 1 to 2×10^8 colony-forming units (CFU)/ml, and then 3 μl of peptide solution was placed over the agar. Incubation time was from 16 to 19 h at 35±2 °C. A halo of growth inhibition was observed as a positive result. Two reference strains were used: *E. coli* ATCC25922 and *S. aureus* ATCC29213. Then, minimal inhibitory concentration (MIC) was determined by using a broth micro-dilution method as indicated by the CLSI recommendations. A 96-well plate was used for bacterial growth in the presence of Müeller-Hinton broth medium (85 μl) and using a peptide final concentration from 0.38 to

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