Biochimica et Biophysica Acta 1820 (2012) 1831–1838

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Antimicrobial peptide pleurocidin synergizes with antibiotics through hydroxyl radical formation and membrane damage, and exerts antibiofilm activity

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ARTICLE INFO

Article history:
Received 11 April 2012
Received in revised form 2 August 2012
Accepted 3 August 2012
Available online 16 August 2012

Keywords:
Pleurocidin
Pleuronectes americanus
Synergistic effect
Antibiofilm effect
Hydroxyl radical
Membrane damage

ABSTRACT

Background: Pleurocidin, a 25-mer antimicrobial peptide (AMP), is known to exert bactericidal activity. However, the synergistic activity and mechanism(s) of pleurocidin in combination with conventional antibiotics, and the antibiofilm effect of the peptide are poorly understood.
Methods: The interaction between pleurocidin and antibiotics was evaluated using checkerboard assay. To study the mechanism(s) involved in their synergism, we detected hydroxyl radical formation using 3′-(p-hydroxyphenyl) fluorescein, measured the NAD^+/NADH ratio by NAD^+ cycling assay, observed change in bacterial viability with the hydroxyl radical scavenger thiourea, and investigated cytoplasmic membrane damage using propidium iodide. Also, the antibiofilm effect of pleurocidin was examined with the tissue culture plate method.
Results: All combinations of pleurocidin and antibiotics showed synergistic interaction against bacterial strains (fractional inhibitory concentration index (FICI) ≤ 0.5) except for *Enterococcus faecium* treated with a combination of the peptide and ampicillin (FICI = 0.75). We identified that pleurocidin alone and in combinations with antibiotics induced formation of hydroxyl radicals. The oxidative stress was caused by a transient NADH depletion and the addition of thiourea prevented bacterial death, especially in the case of the combined treatment of pleurocidin and ampicillin showing synergisms. The combination of pleurocidin and erythromycin increased permeability of bacterial cytoplasmic membrane. Additionally, pleurocidin exhibited a potent inhibitory effect on preformed biofilm of bacterial organisms. In conclusion, pleurocidin synergized with antibiotics through hydroxyl radical formation and membrane-active mechanism, and exerted antibiofilm activity.
General significance: The synergistic effect between pleurocidin and antibiotics suggests the AMP is a potential therapeutic agent and adjuvant for antimicrobial chemotherapy.

1. Introduction

Despite many efforts toward their control and prevention, the emergence of antibiotic-resistant bacteria and the formation of bacterial biofilm associated with chronic infections are clinical problems [1,2]. This generates an urgent need for new antimicrobial agents, and an alternative strategy such as combination therapy [3]. Antimicrobial peptides (AMPs), which are largely diffused in nature, have emerged as potential therapeutic agents, primarily because the development of resistance to them is very low [4]. Also, several studies regarding the synergistic activity of AMPs in combination with conventional antibiotics have been reported. A combination of frog peptide esculentin-1b and polymyxin antibiotic colistin showed a synergistic effect by enhancing the bactericidal activity [5] and interaction between α-helical AMPs and rifampicin achieved reductions in bacterial multiplication, LPS and TNF-α secretion [6].

Bacterial cells adhere to damaged tissue and medical devices including catheters, prosthetic heart valves, artificial hips, and contact lenses and form a slime layer known as a biofilm. Bacterial cells growing in biofilm encase themselves in a self-produced matrix of extracellular polymeric substance (EPS), which can increase antibiotic resistance by up to 1000 fold [7,8]. Dense aggregates of bacteria held together by the diffusion of EPS evade host defenses and antimicrobial chemotherapy [9]. Hence, the antibiofilm effects of AMPs as well as combination therapy have been studied [2,10].

AMPs produced by a wide variety of organisms, are released from secretory glands into internal body fluids or onto mucosal epithelia, and act as the host's first line of defense against invading pathogens [11,12]. Despite displaying extensive sequence variability, most AMPs

Abbreviations: AMPs, antimicrobial peptides; EPS, extracellular polymeric substance; CH_3CN, acetonitrile; LB, Luria Bertani; CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration; NCCLS, National Committee for Clinical Laboratory Standards; FICI, fractional inhibitory concentration index; HPF, 3′-(p-hydroxyphenyl) fluorescein; PES, phenazine ethosulfate; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PI, propidium iodide; TCP, tissue culture plate; BHI, brain–heart infusion

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http://dx.doi.org/10.1016/j.bbagen.2012.08.012

share two important features: a net positive charge and an amphipathic structure. These properties allow AMPs to interact with the anionic microbial surface and to enter into the cytoplasmic membrane [13]. Pleurocidin (GWGSFFKKAAHVGKHVGKAALTHYL-NH_2), a 25-mer AMP with a net positive charge and amphipathic α-helical structure, was derived from the skin mucous secretion of the winter flounder *Pleuronectes americanus* [14]. This peptide has been reported to exhibit a broad antimicrobial activity and induced cell death in yeast via membrane disruption and oxidative stress with almost no hemolysis against human erythrocytes [15–17].

In this study, we investigated the synergism between pleurocidin and conventional antibiotics including ampicillin, chloramphenicol, and erythromycin and its mechanism(s). Additionally, we evaluated the antibiofilm effect of pleurocidin on preformed biofilm.

2. Materials and methods

2.1. Solid-phase peptide synthesis

Anygen Co. (Gwangju, Korea) carried out the peptide synthesis. Anygen Co. offers the following procedures for peptide synthesis. The assembly of the peptides was achieved with a 60-min cycle for each residue at ambient temperature using the following method: (1) the 2-chlorotrityl (or 4-methylbenzhydrylamine amide) resin was charged to a reactor and then washed with DCM and DMF, respectively, and (2) a coupling step was done with vigorous shaking using a 0.14 mM solution of Fmoc-L-amino acids and Fmoc-L-amino acids preactivated for approximately 60 min with a 0.1 mM solution of 0.5 M HOBt/DIC in DMF. Using a TFA cocktail solution, the peptide was cleaved from the resin at ambient temperature. [18,19].

2.2. Peptide characterization

Analytical and preparative reverse-phase HPLC runs were performed with a Shimadzu 20 A or 6 A gradient system. Data was collected using an SPD-20 A detector at 230 nm. Chromatographic separations were achieved with a 1%/min linear gradient of buffer B in A (A = 0.1% TFA in H_2O; B = 0.1% TFA in acetonitrile (CH_3CN)) over 40 min at flow rates of 1 and 8 ml/min using Shimadzu C_{18} analytical (5 μm, 0.46 cm × 25 cm) and preparative C_{18} (10 μm, 2.5 cm × 25 cm) columns. Mass spectrometry was also performed.

2.3. Antibiotics, bacterial strains, and culture conditions

Erythromycin, chloramphenicol, and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecium* (ATCC 19434), *propionibacterium acnes* (ATCC 6919), *Escherichia coli* (ATCC 25922), *E. coli* O-157 (ATCC 43895), and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from the American Type Culture Collection (ATCC). Cells were grown in Luria Bertani (LB) medium (Difco) at 37 °C.

2.4. Antibacterial activity assay

Bacterial strains were cultured in a MH broth and the cell suspensions were adjusted to obtain standardized populations by measuring the turbidity with a spectrophotometer (DU530; Beckman, Fullerton, CA, USA). The bacterial strains at mid-log phase (1×10^6/ml) were inoculated into a MH broth and 0.1 ml was dispensed per well into 96-well microtiter plates. Susceptibility tests were performed by a twofold standard broth-microdilution of the test compounds including pleurocidin, erythromycin, chloramphenicol, and ampicillin, following the Clinical and Laboratory Standards Institute (CLSI) guideline [20]. After 18 h of incubation at 37 °C, the minimal concentration required to prevent the growth of a given test organism was defined as the minimum inhibitory concentration (MIC). The growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, CA, USA) by monitoring optical density at 620 nm (OD_{620}).

2.5. Combination assay

The MICs of each antibiotic agent alone or in combination with pleurocidin were determined by the broth microdilution method in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) using the cation-adjusted Mueller–Hinton broth modified by a checkerboard procedure [21,22]. For the combined treatment, a two-dimensional checkerboard with twofold dilutions of each agent was set up. Growth control wells containing the medium were included in each plate. After 18 h of incubation at 37 °C, the MIC was defined as described above. Each test was performed in triplicate. The interaction of pleurocidin with antibiotics was evaluated by the fractional inhibitory concentration index (FICI). The FICI was calculated according to the equation: $FICI = FIC_A + FIC_B = (MIC_{Drug\ A}$ in combination/$MIC_{Drug\ A}$ alone) + ($MIC_{Drug\ B}$ in combination/$MIC_{Drug\ B}$ alone) [23]. FICI, calculated as the sum of each FIC, was interpreted as follows: $FICI \leq 0.5$ synergy, $0.5 < FICI \leq 4$ no interaction, $4 < FICI$ antagonism [24].

2.6. Hydroxyl radical formation assay

Bacterial cells (1×10^6/ml) were incubated for 2 h at 37 °C each with an agent or in combination with pleurocidin and the final concentration of treated agents was the MIC for agent or MIC for the combination treatments. To detect hydroxyl radical formation, we used the fluorescent reporter dye 3′-(p-hydroxyphenyl) fluorescein (HPF) (Invitrogen) at a concentration of 5 μM [25]. The fluorescence intensity of HPF was measured by a spectrofluorophotometer (Shimadzu RF-5301PC, Shimadzu, Japan) at 490 nm excitation and 515 nm emission wavelengths. The percentage of hydroxyl radical formation was calculated as follows: [(OD of well treated with antibacterial agent − OD of non-treated control/OD of non-treated control) × 100].

2.7. NAD^+, NADH extraction

We performed the dinucleotide extraction and cycling assay by protocols described previously [26]. The bacterial cells at the mid-log phase (1×10^6/ml) were centrifuged at 13,000 rpm for 5 min and resuspended in 1 ml of LB medium. The samples were collected each for NAD^+ and NADH extraction by centrifugation at 13,000 rpm for 1 min from *S. aureus* and *P. aeruginosa* cultures every half hour between 0 and 2 h after adding pleurocidin and ampicillin (at the MIC). The supernatant was removed and the pellets were frozen immediately in a dry ice–ethanol bath and the pellets were stored at −80 °C until we finished collecting all the time-points. 75 μl of 0.2 M NaOH (for NADH extraction) or 75 μl of 0.2 M HCl (for NAD^+ extraction) was added to the ice-cold pellets. The samples were heated at 100 °C for 10 min and subsequently centrifuged at 10,000 rpm for 5 min. The NAD^+/NADH containing supernatants were transferred to fresh tubes and stored in the dark on ice until use.

We performed the NAD^+ cycling assay in 96-well plates. The reaction mixture contained 16 μl of 1.0 M bicine (pH 8.0) (Sigma-Aldrich, St. Louis, MO, USA), 40 μl of sample extract, 40 μl of neutralizing buffer (0.1 M HCl for NADH, or 0.1 M NaOH for NAD^+), 16 μl of phenazine ethosulfate (PES, Sigma), 16 μl of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA), 16 μl of 100% ethanol, and 30 μl of 40 mM EDTA (pH 8.0). We finally added PES and MTT to the 96-well plates and incubated the mixture for 3 min at 30 °C. We added 3.2 μl of alcohol dehydrogenase (500 U/ml, Sigma-Aldrich, St. Louis, MO, USA) in bicine buffer (pH 8.0) to the reaction mixture to begin the assay. The increase in absorbance at 570 nm was recorded within 10 min. The rate of MTT reduction is proportional to the concentration of NAD^+ or NADH in the