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Supporting Information

for

Synergistic Activity of a Short Lipidated Antimicrobial Peptide (LipoAMP) and Colistin or Tobramycin against *Pseudomonas Aeruginosa* from Cystic Fibrosis Patients

by

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Experimental

Peptide synthesis.
Peptide synthesis has been described in details elsewhere.[1] In short, the peptides were synthesized using standard Fmoc-based solid-phase peptide synthesis protocols on an Advanced ChemTech (Louisville, KY) Apex 396 Multiple Peptide Synthesizer. After assembly of the peptide on the resin, the Mtt-protecting group of the N- or C-terminally positioned lysine residue was removed and the resin was divided into several equally portioned batches. To these portions, lipids of different lengths or ferrocene carboxylic acid were attached, after which the N-terminally positioned Fmoc-group was removed. Then, the peptides were detached from the resin using TFA and the appropriate scavengers; during this cleavage of the peptide from the resin, the side-chain protecting groups – i.e. Boc for Trp and Pbf for Arg – were also removed. The cleaved crude peptides were precipitated in cold (-20 °C) Et_2O – hexanes (1:1, v/v) and purified by preparative HPLC (using a gradient of water to MeCN on a C_{18} reversed phase column). The purified fractions were analysed by analytical HPLC and ESI-MS in order to confirm the purity and identity of the obtained derivatized peptides. Generally, all peptides were obtained in 21–46% yield, in >10 mg quantity, and with >95% purity. Characterization of the compounds is given in reference 25, that of the new peptide BA250-DEC is: RP-HPLC (C_{18}-column, buffers as described before in 25): t_R = 19.9 min. ESI-MS: 1416.83 (calc. 1416.65 for $[M+H]^+$, M = $C_{71}H_{94}N_{16}O_{10}$).

Antibiotics.
Stock solutions were prepared according to the guidelines of the National Committee for Clinical Laboratory Standards.[2] Six conventional anti-pseudomonal antibiotics were used: tobramycin, ciprofloxacin, colistin (used as colistin sulfate), ceftazidim, tazocin, and meropenem. The antibiotics were solubilized in NaCl 0.9 % or in sterile water.

Bacterial strains and culture.
Three clinical isolates were used to determine the optimal configuration of the lipoAMP, i.e. C- versus N-terminal lipid, lipid-length versus organometallic moiety (Table 1). For this, the peptides were dissolved in DMSO at a concentration of 10 mg/mL, stored at –20 °C, and used within two weeks. Twenty *P. aeruginosa* strains, isolated from sputum of CF patients, were used for the experiments (Table 2). Strains were stored as a stock at −80 °C. For each set of experiments, bacteria were streaked onto a (TAS) agar medium plate and cultured for 18–22 h at 37 °C. An individual colony was cultured in cation-adjusted Mueller Hinton broth (CAMHB; Mueller Hinton broth supplemented with Ca^{2+} (20–25 mg/L) and Mg^{2+} (10–12.5 mg/L)) or LB medium containing 1% glucose for the biofilm assay to prepare aerated, log-phase bacteria by rotary shaking at 37 °C. Spectrophotometry was performed and the overnight culture was diluted to an OD_{600} of 0.01.

Minimal Inhibitory Concentration (MIC).
The MIC was determined by standard microdilution of the antibacterial agent according to CLSI 2007 recommendations, using CAMHB[3] and 96-well plates. The overnight culture in CAMHB medium was diluted to an OD_{600} 0.01. After re-growing to an OD_{600} of 0.5 cells were diluted and 10^5 cells in 100 µL were added to the wells. MIC values were determined after 16–20 h at 37 °C. The concentration at which no growth was seen was set as the MIC (determined in duplicate, technical and biological).

MIC determination of combination of BA250-C10 and colistin or tobramycin using the checkerboard combination assay.
In the combination assays, the 'Checkerboard' procedure described by White was followed.[4] This method allows varying the concentrations of two antimicrobial compounds along the different axes, thus ensuring that each well contained a combination of different concentrations for the two antibiotics tested. In this method, a reduction in the MIC value of each compound in the presence of the other indicates synergy. The combination assays were performed in a sterile 96-well plate containing CAMHB with the respective antibiotic serially diluted on the x-axis, and increasing concentrations of BA250-C10 on the y-axis. Diluted cell cultures were then added (using an overnight culture, diluted to an OD_{600} of 0.01 AU), after which the plate was cultured for 18–22 h at 37 °C. Then, growth inhibition was measured by automated reading of bacterial densities with a standard microtiter plate reader at 600 nm.

Following our initial results from the checkerboard assay, in which colistin and tobramycin showed synergistic effects, these two antibiotics were selected for further evaluation. Also, colistin and tobramycin are important antibiotics when it comes to the treatment of *P. aeruginosa* infections in CF patients. Four clinical isolates were selected for evaluation by the combination assays:

- Pa01: main reference isolate;
- clone C: a representative of a *P. aeruginosa* clonal lineage, which is highly occurring among CF patients in Europe;
- LESB58: resistance against 5 antibiotics, including colistin;
- KD491: resistance against 5 antibiotics, forms a biofilm.

The checkerboard assays were performed in duplicate. To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) was calculated for each antibiotic in each combination.[5] The FIC is identified as the ratio of the concentration of the peptide in an inhibitory concentration (i.e. <20% of bacterial growth of the control) with or without the antibiotic, and *vice versa*. The FIC provides a measure of the degree of synergy between two antimicrobial agents against a particular microorganism. FIC values of 0.5 or below are labeled as 'synergism', values just above 0.5 indicate only a 2-fold change in the MIC-value for one agent and a larger change in the MIC for the other; they are labeled as 'indifference'.[6] The following formulae were used to calculate the FIC index: FIC of antibiotic (ab) (i.e. "FIC_{ab}") = (MIC of ab in combination with BA250-C10) / (MIC of ab alone). FIC of BA250-C10 (i.e. "FIC_{AMP}") = (MIC of BA250-C10 in combination with ab) / (MIC of BA250-C10 alone), and FIC index= FIC_{ab} + FIC_{AMP}.

Growth curves.

Growth curves were performed using three different (mucoidal) strains: KD491, Pa01, and clone C, in 10 x 10 bioscreen plates. After dilution of the overnight cultures in CAMHB to 0.005 OD_{660}, 150 µL was added to a 150 µL dilution of BA250-C10 alone, or in combination with tobramycin or colistin. Automated reading of bacterial densities in the bioscreen plate was done every 15 minutes using a standard microtiter plate reader at OD_{600}.

Biofilm susceptibility assay.

Biofilm-formation was evaluated by the polystyrene assay using crystal violet to stain the biofilm.[7] In this assay, 1 mL of fresh medium was prepared with different concentrations BA250-C10, tobramycin, colistin, or with BA250-C10 in combination with tobramycin or colistin. First, the isolates were grown overnight in LB medium and then added to the fresh medium to obtain an OD_{660} of 0.01. Six wells of a flat-bottom 96-well polystyrene microtiter plate (Corning) were then filled with 100 µL of the same solution. All strains were tested in fresh medium for their biofilm forming capacity. Different media were tried in order to induce maximum biofilm formation (LB, LB with 1% glucose, caMHB, M63 (with or without Casamino Acids 0.5%). For the tests, KD491 was grown in LB glucose 1%, and Pa01 and Clone C were grown in M63 medium (0.2% glycerol, 1 mM $MgSO_4$, 0.2% glucose and 0.5% Casamino Acids). The plate was then cultured for 18–22 h at 37 °C and planktonic growth was measured by automated reading of bacterial densities with a standard microtiter plate reader at 600 nm. After removing the planktonic cells and washing twice with PBS, the plate was dried for an hour (at 55 °C) to improve attachment of the biofilm. For staining of the biofilms the wells were filled with a 0.2% crystal violet solution and incubated for 15 minutes before being washed two times with PBS. Then 170 µL acetic acid (30 %, v/v, in water) was added to solute the crystal violet. After 10 minutes the optical density was measured in a standard microtiter plate reader at 600 nm. The percentage of inhibited biofilm was calculated as follows: ((1 – (OD_{600} treated / OD_{600} non-treated) × 100. Experiments were done in duplicate with six technical replicates.

Biofilm formation in a semi-static model.

Biofilms were grown in LB+ 1% glucose with or without BA250-C10 (8 µg/mL) on a cover slip coated with poly-L-lysine (0.45 µm; diameter, 12 mm; Becton Dickinson) inside a well from a six well polystyrene plate (Corning Inc.) at 37 °C for 24 h at 120 rpm. After 24 h, biofilms were chemically fixed in 8% glutaraldehyde (Merck) for 20 min and stained with 15 µg/mL propidium iodide (PI) for 15 min. After staining, biofilms were analyzed by confocal laser microscopy (Leica SP5) with a DFC360 FX Digital Camera Kit SP5 (Leica), equipped with an oil plan-neofluor 63x/1.4 objective. PI was excited at 633 nm. Z-stacks were taken with an interval of 0.42 µm. Pictures were analyzed with LAS AF software (Leica).

References for the Supporting Information:

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