Supporting Information

Porphyrin-Apidaecin Conjugate as a New Broad Spectrum Antibacterial Agent

Ryan Dosselli,[†] Marina Gobbo,[‡] Erika Bolognini,[‡] Sandro Campestrini,[‡] and Elena Reddi[†]*

[†]Department of Biology, University of Padova, via U. Bassi 58/B, 35131 Padova, Italy and
[‡]Department of Chemical Sciences, University of Padova, via Marzolo 1, 35131 Padova, Italy

Table of contents:

Chemicals
Fmoc-Leu-Wang (200-400 mesh, loading 0.6 mmol/g resin) was purchased from Novabiochem (Merck Biosciences, La Jolla, CA, USA). Fmoc-amino acids and reagents for peptide synthesis were supplied from Sigma-Aldrich Co. (St. Louis, MO, USA). 5-Carboxy-5,10,15,20-tetraphenylporphyrin (cTPP) was synthesized according to the literature [Matile S.; Berova, N.; Nakanishi, K.; Fleischhauer J.; Woody, R. W. Structural studies by exciton coupled circular dichroism over a large distance: porphyrin derivatives of steroids, dimeric steroids and brevetoxin B *J. Am. Chem. Soc.* **1996**, 118, 5198-5206].

Synthesis of the porphyrin-apidaecin conjugate
The peptides were synthesized by the solid phase procedure on a Advanced Chemtech 48Ω Multiple Peptide Sinthetizer on a 0.12 mmol scale, starting from a pre-loaded Fmoc-Leu-Wang resin. Trifunctional amino acids were protected on the side chain as Arg(Pbf), Asn(Trt), Gln(Trt), His(Trt) and Tyr(*t*Bu). Fmoc amino acids (4 eq) were coupled by the standard HBTU/HOBt/DIEA protocol and in the synthesis of the porphyrin conjugate, cTPP (2.5 eq) was activated by DIC/HOBt. Peptides were cleaved from the resin and simultaneously deprotected with a trifluoroacetic acid-water-triisopropylsilane mixture (95 : 2.5 : 2.5 by volume). Crude peptides, precipitated by addition of cold diethyl ether, were obtained in 70-80% yield. After purification by semi-preparative HPLC [Shimadzu series LC-6A chromatographer; column: Vydac C_{18} (250 x 22 mm, 10 μm, flow rate at 15 mL/min); mobile phase A (aqueous 0.1% TFA) and B (90% aqueous acetonitrile, 0.1% TFA); elution condition: isocratic 45% B for 5 min; 45-75% B in 20 min], apidaecin and the TPP-apidaecin conjugate were characterized by analytical HPLC [Dionex Summit Dual-Gradient HPLC; column Vydac C_{18} (250 x 4.6 mm, 5 μm, flow rate at 1.5 mL/min); mobile phase as above; elution condition: *gradient* ***a***: isocratic 10% B for 3 min; 10-90% B in 20 min; *gradient* ***b***: isocratic 10% B for 3 min; 10-50% B in 20 min; *gradient* ***c***: isocratic 20% B for 3 min; 20-70% B in 20 min] and ESI-MS (Mariner API-TOF Workstation).

Apidaecin Ib: HPLC: t_R 10.90 min (***a***), t_R 13.60 min (***b***); ESI-MS: $[M+H]^+$ calc. 2108.16, obs. 2108.18

cTPP-apidaecin: HPLC: t_R17.07 min (***a***), t_R 19.52 min (***c***); ESI-MS: $[M+H]^+$ calc. 2748.38, obs. 2748.34.

UV-visible absorption spectra
UV-visible absorption spectra of cTPP and T-api were recorded at room temperature with a Perkin-Elmer spectrophotometer (model Lambda 5, Norwalk, CT) and using 1 mm optical path quartz cuvettes.

Bacterial strains
The methicillin-resistant strain of *Staphylococcus aureus* ATCC BAA-44, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 25668 were purchased from LGC Promochem (Teddington, UK). Cultures were maintained by two weeks' subcultures in brain heart infusion (BHI; Difco, Detroit, MI) agar.

Determination of MIC
The minimal inhibitory concentration values (MICs) of apidaecin, T-api and cTPP were determined by the broth microdilution susceptibility test following the guidelines of the NCCLS with mid-log phase cultures. Serial two-fold dilutions of apidaecin, T-Api and cTPP were prepared (final volume of 50 µl) in 96-well polypropylene microtiter plates (Sarstedt, Germany) with 50% Muller Hinton (MH; Difco, Detroit, MI) broth in phosphate buffered saline (PBS, 10 mM phosphate, 0.14 M NaCl, 2.7 mM KCl, pH 7.3). Each dilution series included control wells without peptide. A total of 50 µl of the adjusted inoculum (approximately 2.5×10^5cells/mL) in 50% MH broth was added to each

well. To evaluate the MIC, microtiter plates with bacteria were incubated at 37 °C overnight, and the plates analyzed with an ELISA plates analyzer (Lp 200 Diagnostic Pasteur). The MIC value was defined as the lowest concentration of compound that prevented visible bacterial growth after incubation for 18 h at 37 °C.

Photoinactivation of bacteria

For the photoinactivation experiments, the bacteria were grown overnight in BHI at 37 °C; harvested by centrifugation; washed twice and resuspended in PBS at a density of ~ $2x10^7$ cells/mL. The cell density was evaluated by measuring the turbidity of the suspension at 650 nm in a Perkin-Elmer spectrophotometer (model Lambda 5, Norwalk, CT). The bacteria used in the experiments were collected from cultures in the stationary phase of growth.

The bacteria were incubated with cTPP, apidaecin or T-Api in the dark at room temperature for 60 min. After incubation, the suspensions were: i) directly exposed to light with the unbound cTPP, apidaecin or T-api left in the suspension (no washing); ii) centrifuged (1,180 × g for 15 min), the pellet resuspended in 1 mL of PBS and illuminated (1 washing); iii) centrifuged and the cell pellet washed two additional times with PBS before illumination (3 washings). For illumination, aliquots of cell samples obtained as described above were transferred into 96-well plates (200 µL/well). The light source was the UV 236 lamp supplied by Waldmann Eclairage SA (Reichstett, France). The light emission of the lamp was 390-460 nm with the maximum at 420 nm and width of the half-maximum of 35 nm. The cells were illuminated from the bottom of the plates for 20 min with a fluence rate of 15.2 mW/cm^2, as measured with the Waldmann UV-meter, and for a total light dose of 13.4 J/cm^2. After illumination, aliquots of bacteria suspensions were serially diluted 10-fold in PBS and 50 µL of appropriate dilutions were plated in duplicate onto BHI agar to determine colony forming units (cfu). Suspensions of bacteria exposed to apidaecin, cTPP and T-api but kept in the dark and subjected to the same procedure applied to the irradiated suspensions were also plated onto BHI agar after appropriate serial dilutions. Controls included also bacteria not exposed to any agent and bacteria exposed to light (13.4 J/cm^2) only. Treated and untreated cells were incubated overnight at 37 °C to allow colony formation.

Fluorescence microscopy. For the fluorescence microscopy experiments, the bacteria were grown and treated in the same conditions used for photoinactivation experiments. Only the higher dose of either T-api or cTPP used in the photoinactivation experiments for each bacterial strain was tested. After incubation with photosensitizer, bacteria were washed once (1 washing) or three times (3 washings) with PBS, and the resulting pellets resuspended in 10 µL of PBS and analyzed by fluorescence microscopy with a Leica DM 5000b microscope. Images were acquired in bright field and fluorescence mode using a custom filter with excitation at 420 ± 20 nm and emission at 655 ± 20 nm.