Title: OH Radical Production Stimulated by $(RW)_{4D}$, A Synthetic Antimicrobial Agent and Indolicidin

Zhigang Liu [a†], Yi Cai [a,b†], Anne W. Young [a†], Filbert Totsingan [a], Nikhil Jiwrajka [a], Zhengshuang Shi [a,b #] and Neville R. Kallenbach [a #]

[a] Department of Chemistry, New York University, New York, NY 10003, USA
[b] School of Chemistry and Chemical Engineering, Huazhong University of Science and Technology, Wuhan 430074, China

[†] These authors contributed equally to the work.
[#] Correspondence should be addressed to NRK or ZS

Mailing address: School of Chemistry and Chemical Engineering, Huazhong University of Science and Technology, 1037 Luoyu Road, Wuhan 430074, China. Phone: 86-27-8779-3907. Fax: 86-27-8754-3632. Email: zs_shi@mail.hust.edu.cn or Department of Chemistry, New York University, 100 Washington Square East, New York, NY 10003. Phone: (212)-998-8757. Fax: (212)-260-7905. Email: nrk1@nyu.edu

Electronic supplementary information (ESI)

Materials and Methods

Preparation of ILN and $(RW)_{4D}$

ILN was assembled on Rink Amide AM resin from Nova Biochem (San Diego, CA) using Fmoc (9-fluorenylmethoxycarbonyl) chemistry. Cleavage of the peptides from the resin was performed with 95% trifluoroacetic acid (TFA) in the presence of the scavenger, 2.5% triisopropylsilane (TIS), and 2.5% water. After precipitation with cold ether, samples were purified by reverse-phase high-performance liquid chromatography (HPLC) C_{18} preparative column (2.2 by 25 cm, 300 Å; Grace Vydac Co., Hesperia, CA) using water and acetonitrile as eluants. Fractions containing product were pooled and lyophilized. The molecular weight of the peptide was confirmed by a Bruker matrix-assisted laser desorption ionization-time-of-flight mass spectrometer (Billerica, MA).

$(RW)_{4D}$ ($(RW)_4$-K_2K-β-Ala-NH_2, Scheme 1) were synthesized using the same Fmoc peptide synthesis strategy, except Fmoc-Lys(Fmoc)-OH was used instead of normal Fmoc-Lys(tBoc)-OH to give branched reactive amines for multivalent designs as described in reference [3].

Hydroxyl Radical Assay

Gram-negative *E. coli* MG1665 and its mutants (ΔiscS, ΔrecA, ΔacnB, Δmdh, ΔsucB, and ΔicdA) were purchased from ATCC. Three bactericidal antibiotics- norfloxacin, ampicillin, kanamycin- and three bacteriostatic drugs- chloramphenicol, spectinomycin and tetracycline- were purchased from Sigma. Hydrogen peroxide (H_2O_2) was also from Sigma. Thiourea was obtained from Fluka (St. Louis, MO). The OH sensitive fluorescent reporter dye 3′-(p-hydroxyphenyl) fluorescein (HPF) was purchased from Invitrogen.

MG1665 and mutant cells were picked as single colonies and cultured in LB broth until they reached early exponential growth (OD600 ~ 0.3) at 37˚C with shaking at 300 rpm. The bactericidal/bacteriostatic antibiotics at respective low and high concentrations (norfloxacin (25ng/mL and 250ng/mL), ampicillin (5μg/mL and 15μg/mL), kanamycin (5μg/mL and 25μg/mL), chloramphenicol (7μg/mL and 15μg/mL), spectinomycin (200μg/mL and 400μg/mL) and tetracycline (4μg/mL and 10μg/mL)) or $(RW)_{4D}$ (9μg/mL and 45μg/mL) or indolicidin (8μg/mL and 40μg/mL) or 1mM hydrogen peroxide were then added to the culture for 1 hr or 2 hrs as indicated. To quench hydroxyl radical, thiourea was added to the culture to achieve a final concentration of 150mM in solution at the same time as the drugs were applied. Bacterial cells were harvested and washed with cold PBS. Cells were stained in a HPF/PBS solution diluted 1:1000 at room temperature for 30 min while being constantly shaken. Cells were then washed twice and subjected to flow cytometry acquisition and analysis using a FACScan (BD Biosciences, Mountain View, CA) equipped with a 488 nm argon laser and a 515–545 nm emission filter (FL1) at low flow rate and photomultiplier tube (PMT) voltage settings at E00 (FSC), 360 (SSC), and 825 (FL1). At least 50,000 cells were collected for each sample. Flow data were analyzed with FlowJo software (TreeStar).