Electronic Supplementary Information

Materials and Methods

Synthesis of Linear Peptide

Linear sequence $(RW)_4$-NH_2 was assembled on Rink amide resin from Nova Biochem (San Diego, Calif.) with a RAININ Instrument PS3 solid phase synthesizer (Woburn, Mass.) using Fmoc chemistry. Fmoc-Trp(Boc)/Arg(Pbf), the coupling reagent HBTU and HOBT were also purchased from Nova Biochem. Cleavage of peptides from the resin was performed with 95% TFA in the presence of the 2.5% TIS and 2.5% H_2O. After precipitation with cold ether, samples were purified on a reverse-phase HPLC C-18 preparative column (2.2 x 25 cm, 300Å, Grace Vydac Co., Hesperia, Calif.) with water and acetonitrile as elution solvents. Fractions containing product were pooled and lyophilized. The molecular weight of the peptide was confirmed by a Bruker MALDI-TOF spectrometer (Billerica, Mass.), which is in agreement with theoretical masses. $(RW)_4$: $M+H^+$ calculated 1386.6, found 1386.7.

Synthesis of Dendrimeric Peptides

Rink amide resin and Fmoc chemistry were used in the synthesis of the dendrimeric AMPs. First, an Fmoc-protected β-alanine residue, making up the C-terminus, was coupled using coupling reagent HBTU (2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyluroniumhexafluoro phosphate) and HOBT (N-hydroxybenzotriazol) (Nova Biochem). Using the same protecting group for both amino groups allows for simultaneous deprotection, so that two amide bonds are formed during the next coupling step. Tetravalent and octavalent cores were achieved by repeating this step. Then dipeptide variations were simultaneously coupled to NH_2 groups of the tetravalent template composed of 3 residues of lysine. For the synthesis of the dendrimer $(RWRW)_{4D}$, $(RWRWRW)_{4D}$, and $(RWRW)_{8D}$, 4 or 8 (RW)n (n=2, 3) were simultaneously coupled to NH_2 groups of the lysine core templates. Crude peptide solutions were deprotected by 95% trifluoroacetic acid (TFA), in the presence of the scavenger 2.5% triisopropylsilane (TIS), and 2.5% H_2O. It was precipitated in ether and purified on reverse phase HPLC. Molecular weights were verified by M/S using a Bruker MALDI-TOF spectrometer, which were in agreement with theoretical masses. $(RW)_4$-Lys_2-Lys-Beta-Ala-NH_2: $M+2H^+$ calculated 1843.0, found 1843.0; $(KW)_4$-Lys_2-Lys-Beta-Ala-NH_2: $M+2H^+$

calculated 1789.1, found 1789.5; $(HW)_4$-Lys_2-Lys-Beta-Ala-NH_2: $M+H^+$ calculated 1646.9, found 1646.6; $(RY)_4$-Lys_2-Lys-Beta-Ala-NH_2: $M+H^+$ calculated 1750.0, found 1750.5; $(KY)_4$-Lys_2-Lys-Beta-Ala-NH_2: $M+H^+$ calculated 1632.0, found 1632.6; $(RF)_4$-Lys_2-Lys-Beta-Ala-NH_2: $M+H^+$ calculated 1686.0, found 1686.5; $(KF)_4$-Lys_2-Lys-Beta-Ala-NH_2: $M+H^+$ calculated 1696.0, found 1696.5; $(RWRW)_4$-Lys2-Lys-Beta-Ala-NH_2: $M+4H^+$ calculated 3381.8, found 3380.0; $(RWRWRW)_4$-Lys2-Lys-Beta-Ala-NH_2: $M+4H^+$ calculated 4750.5, found 4750.5; $(RWRW)_8$-Lys_4-Lys_2-Lys-Beta-Ala-NH_2: $M+4H^+$ calculated 6799.7, found 6801.8.

Preparation of Tetrapeptide RWRW with side chain protected

Peptides R(Pbf)W(tBoc)R(Pbf)W(tBoc)-NH_2 were assembled on Sieber Amide resin (Nova Biochem, Corp., CA) by using the same synthesis as above but without capping N terminus at the last step of synthesis. After completion of synthesis, peptides were cleaved from resin with 1% TFA in dichloromethylene. This gave the peptide product with side chain-protecting groups on to avoid cross-linking in further reactions. Crude product containing > 95% of the expected peptide, according to analytical HPLC, was used for preparation of multivalent peptides without further purification. LC/MS *m/z* for R(Pbf)W(tBoc)R(Pbf)W(tBoc)-NH_2 $[M]^+$ calculated 1406.66, found 1407.3.

Preparation of polydisperse multivalent AMPs

The PMA samples were supplied with an average molecular weight of 5000 (according to the manufacturer, Polysciences, Inc., PA), corresponding to an average of 50 maleic anhydride units per polymer chain. The conjugation reaction between PMA (15mg, 50mg/ml) and 100 equivalent excess peptides (50mg, 100mg/ml) was carried out overnight at 40 °C in DMF with agitation on a rotary shaker catalyzed by diisopropylethylamine (DIEA) (100 equiv). The unreacted maleic anhydride groups were quenched by the addition of 150 µl distilled and deionized water, and then the product was treated with excess trimethylsilyldiazomethane (TMDM) to methylate the resulting carboxylic acid groups. After final solutions were dialyzed overnight (5000 mwco, 1000 mL) in distilled water, the polymeric peptides were isolated. Similar conditions were used to generate partially grafted polymeric peptides 50% and 25%. Control polymers were generated by the addition of 100 equivalent excess methylamine solution in THF (Sigma-Aldrich Co., MO) instead of peptides and isolated by steps described above. The cleavage

of side chain-protecting groups from the polymer was performed with 95% TFA, 2.5% TIS and 2.5% H_2O. After precipitation with cold ether and overnight lyophilization, final polymer products were obtained without further purification. The composition of the product was confirmed by ^{1}H NMR (Bruker 400 MHz, acetonitrile-d_3). PMA-RWRW, δ1.40 (m, 2H, $R_\gamma H$), δ1.80 (m, 2H, $R_\beta H$), δ2.0~2.4 (m, nH, PMA (CH- CH)$_n$), δ2.90 (m, 2H, $R_\delta H$), δ3.10 (m, 2H, $W_\beta H$), δ3.50~3.70 (S, 3H, CO_2Me), δ4.05 (m, 1H, $R_\alpha H$), δ4.30 (m, 1H, $W_\alpha H$), δ6.6~7.4 (R $N_\varepsilon H$, H in W ring, terminal amide) δ7.70 (d, 1H, R NH), δ8.10 (d, 1H, W NH), δ9.95 (d, 1H, W $N_\varepsilon H$). PMA control, δ2.0~2.4 (m, nH, PMA (CH-CH)$_n$), δ2.6-2.9 (m, 3H, *N*-CH_3), δ3.50~3.75 (S, 3H, CO_2Me), δ8.0 (m, 1H, NH).

Characterization of polydisperse multivalent AMPs

The extent of substitution on each polymer was estimated by integration of ^{1}H NMR peaks from the multivalent peptides. NMR samples were prepared by dissolving about 5 mg of product into 0.6 mL acetonitrile-d_3. The NMR resonances used for composition determination were peak A at δ1.80 ppm (assigned to be βCH_2 of Arg residues in peptides) and peak B at δ3.50~3.70 (assigned to be CO_2Me). The integrals of these two peaks were used to determine the extent of substitution, giving the results of calculated molecular weight and monomer content in PMA in Table 1. For control polymers, proton chemical shifts δ2.6-2.9 (assigned to be *N*-methyl) and δ3.50~3.75 (assigned to be CO_2Me) were used to determine the extent of reaction ratio. The polymers are moderately polydisperse, with a polydispersity index > 2, measured by light scattering (Dynapro, Protein Solutions, Ltd., UK). The molecular weight distribution of each of the polymers was determined by gel permeation chromatography system including a Beckman Coulter 127S solvent module pump, model 166 absorption detector and a calibrated TSK-GEL column (7.8 × 300 mm) packed with SDVB (Styrene Divinyl Benzene) for size-based separations (Tosoh Bioscience, LLC., CA). Polystyrene samples (Polymer Laboratories, Inc., MA) of different molecular weight were used as standards and THF at a 1.0 mL/min flow rate was used as a mobile phase throughout the analysis. The peak average molecular weight (Mp) of the polymeric peptides relative to standards is listed in Table 1 without further calibration.

Preparation of multivalent AMPs with linear Ac-(GK)$_{4/8}$-NH_2 scaffold

The synthesis of brush-like multivalent AMPs started with forming the peptide with sequence Ac-(GK(mtt))$_{4/8}$-NH_2. After the N terminus of each peptide was capped with acetic anhydride, side chain protecting groups Mtt of Lys-K were cleaved with 1% TFA in dry dichloromethylene while the peptide remained on the solid matrix, which will yield 4/8 free amino groups for the next incoming amino acid. Then the parallel synthesis of 4/8 monomeric RWRW was performed on the amino groups of the lysine side chain by using Fmoc chemistry. Crude peptide solutions were deprotected by 95% TFA, 2.5% water, 2.5% TIS, precipitated in cold ether and purified on reverse phase HPLC. Molecular weights were verified by M/S using a Bruker MALDI-TOF spectrometer. The results are in agreement with theoretical masses. Ac-[GK(-RWRW)]$_4$-NH_2: $M+H^+$ calculated 3706.0, found 3706.0; Ac-[GK(-RWRW)]$_8$-NH_2: $M+H^+$ calculated 7352.0, found 7354.0.

Antimicrobial and hemolytic assays

The antimicrobial activity of each peptide was tested following standard broth microdilution protocols recommended by the National Committee for Clinical Laboratory Standard[1]. Multidrug-resistant *Staphylococcus aureus* (ATCC BAA-44) and *Acinetobacter baumannii* (ATCC BAA-747) were obtained from the American Type Cultures Collections (ATCC; Rockville, MD) and used in this study. Ampicillin- and streptomycin-resistant *Escherichia coli* (D31), were obtained from the E. coli Genetic Resource Center (Yale University, New Haven, CT). Bacteria were grown overnight in Tryptic Soy broth (TSB) at 37°C. Cultures were diluted in Mueller-Hinton broth (MHB) to a final concentration range of 2 x 10^4 to 2 x 10^5 CFU/mL. Diluted bacterial cultures were then mixed with a small fixed volume of phosphate-buffered saline (PBS) buffer (pH 7.2) in which varying peptide stock solution was added. Bacterial cultures were then incubated at 37°C for 18 hours. The 18-hour absorbance data were used to calculate the percentage of inhibition for each sample by comparison with the absorbance of cultures without sample. Bacterial growth was measured by turbidity as the optical density at 600 nm (OD_{600}), using a Genesys 5 Spectrophotometer (Rochester, NY). All assays were carried out in triplicate. The concentration of compound that resulted in 50% inhibition of growth was recorded as the IC_{50} (Table 2).

Hemolytic activity was assessed on fresh sheep erythrocytes (Fitzgerald Inc., Concord, Mass). The red blood cell suspension was incubated in PBS buffer (pH 7.2) with varying concentrations of peptide stocks at 37°C for 60 minutes, and then centrifuged at 3,000 rpm for 10 mins. The resulting supernatant was diluted by a factor of 40 in distilled water. The absorbance of the supernatant at 540 nm was measured using a Genesys 5 Spectrophotometer. Zero hemolysis and 100% hemolysis controls were obtained by incubating the cells with PBS buffer and 1% Triton-X, respectively (Table 2). Peptide concentrations yielding 50% hemolysis were used as the hemolytic dose $(HD)_{50}$ determined from dose-response curves (Table 2).

Resistance Assays

We determined the ability of Gram-negative *A. baumannii* and *E. coli* D31 and Gram-positive *S. aureus* to develop resistance against $(RW)_{4D}$, $(RW)_4$, indolicidin (Anaspec, Inc), vanomycin, chlorhexidine, and gentamicin (Sigma-Aldrich). Bacteria were grown overnight in TSB at 37°C. Then, the cultures were diluted in MHB to a final concentration range of 2×10^4 to 2×10^5 CFU/mL. Diluted bacterial cultures were then mixed with a small fixed volume of phosphate-buffered saline (PBS) buffer (pH 7.2) in which varying peptide stock solution was added. Bacterial cultures were then incubated at 37°C for 18 hours. The 18-hour absorbance data were used to calculate the percentage of inhibition for each sample by comparison with the absorbance of cultures without sample. Bacterial growth was measured by turbidity as the optical density at 600 nm (OD_{600}), using a Genesys 5 Spectrophotometer (Rochester, NY). The concentration of the compound that resulted in 50% inhibition of growth after the first day was used to determine the $IC_{50\ initial}$.

After the $IC_{50\ initial}$ was determined, each agent was incubated at a series of sub-lethal concentrations in the presence of bacteria inocula (2×10^4 to 2×10^5 CFU/mL). The OD_{600} was measured every 10 generations to determine the current IC_{50} until it reached a stationary phase at 20 generations at which point the cultures were subcultured, and washed with PBS to remove any residual agent. Cultures were then diluted to 2×10^4 to 2×10^5 CFU/mL for each bacterial inoculum and the study was done continuously until 400 generations were reached. The emergence of resistance during the 400 generation period

in the presence of the various agents was determined by its relative IC, which is the ratio of IC obtained for a given subculture to $IC_{50\ initial}$.

Reference:

1. NCCLS. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M100-S14*. National Committee for Clinical Laboratory Standards: Wayne, PA., 2004.