Peptide design, synthesis and characterization

Hydrophobicity index scale used for calculating mean per residue hydrophobicity and amphipathicity

The amino acid hydrophobicity index scale used for determining the mean per residue hydrophobicity and amphipathicity of these peptides (Table 2) was developed *ad hoc* as a normalized and filtered consensus of 163 published scales [1], and is arbitrarily ranged between values of +10 for Phe, and -10 for Arg. It was possible to extrapolate index values for the non-proteinogenic amino acid residues as this scale correlates very well with both the octanol/water partition coefficients [2] and reversed-phase retention times for amino acids, as described previously [1]. The index values for amino acids used in this study were: Abu (1.7); Acp (6.2); Aib (1.1); Ala (-1.1); Dab (-9.3); Dap (-9.5); Deg (6.5); Dpg (13.7); Hse (-3.5); Gln (-6.0); Gly (-2.4); Lys (-9.9); Nle (9.1); Nva (5.3); Orn (-9.0); Ser (-4.3); Tyr (2.5). The mean hydrophobic moment was calculated as described by Eisenberg *et al.* [3]. The amphipathicity of peptides is given relative to the maximum possible value (μH_{max} = 6.4 with our scale) for a perfectly amphipathic, 18-residue peptide composed only of Phe and Arg, thus having a relative amphipathicity ($\mu H/\mu H_{max}$) value of 1. This relative measure of amphipathicity is more universal than an absolute value, which varies according to the scale used.

Peptide synthesis and characterization

Solid phase peptide syntheses were performed with the column thermostatically maintained at 50 °C and loaded with PEG-PS resin (substitution 0.17–0.22 meq/g) for all peptides but the one containing Dpg which was carried out manually. The details are given in the appendix. An eightfold excess of 1:1:1.7 Fmoc-amino acid/TBTU/DIPEA was used as default for each coupling step. Double coupling with PyBOP was carried out for all α-branched amino acid residues and HATU was used as activator for the following residue. Peptides were cleaved from the resin and deprotected with a trifluoroacetic acid/water/triisopropylsilane mixture (95:2.5:2.5, by vol.) and purified by preparative RP-HPLC (Waters Delta-Pak® C_{18}, 15 µm, 300 Å, 25 mm × 100 mm), using a 0-60 % (v/v) acetonitrile/water gradient with 0.05 % TFA. The correctness and purity of peptides were determined by analytical RP-HPLC (Waters Symmetry® C_{18}, 3.5 µm, 100 Å, 4.6 mm × 50 mm), followed by mass determination [electrospray ionization-MS, Perkin Elmer/Sciex API *I,* PE Biosystems, Foster City, CA, U.S.A.]. Peptide concentrations were determined based on the molar

absorption coefficient of tyrosine (ε_{280} = 1290 $M^{-1}cm^{-1}$) and confirmed using the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.).

CD spectra were obtained on a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) using 2-mm path-length quartz cells and peptide concentrations of 40 μM, in 5 mM SPB pH 7.0, in the presence or absence of 50 % (v/v) TFE and SDS micelles (10 mM SDS in buffer). The percentage helicity was determined as $([\theta]_{meas} - [\theta]_{rc})/ ([\theta]_{\alpha} - [\theta]_{rc})$, where $[\theta]_{meas}$ is the measured ellipticity at 222 nm, $[\theta]_{rc}$ is the ellipticity for unstructured peptide in the absence of additives (generally close to zero), and $[\theta]_{\alpha}$ is the ellipticity of a fully structured helix with *n* peptide bonds, calculated using the relation $[\theta]_{\alpha} = 39000\ (1\text{-}4/n)$ [4]. The effect on ellipticity due to the presence of achiral α-branched amino acids was taken into account in these calculations.

SPR

Chip preparation and SPR measurements

After rinsing the L1 chip overnight with Milli-Q water and then washing with the non ionic detergent, OG (40 mM, 25 μl, 5 μl/min), SUV suspensions (80 μl, 0.5 mM) of PC/cholesterol (10:1, w/w) or PE/PG (7:3, w/w) were applied to the chip surface at a low flow rate (2 μl/min). This was then increased to 50 μl/min, to remove any multilamellar structures, until a stable baseline was obtained. BSA (25 μl, 0.1 mg/μl in PBS) was used as negative control to confirm complete coverage of non-specific binding sites.

Peptide solutions (20-40 μl PBS solutions of 0.45 - 15 μM peptide) were injected on the lipid surface at a flow rate of 5 μl/min, followed by PBS alone for 30 min to allow peptide dissociation.

Rate equations

The affinity constant was determined using kinetic models suitable for peptide-lipid interactions, by curve fitting using numerical integration analysis [5].

The differential rate equations for two-state reaction model are represented by:

$$dRU_1/dt = k_{a1} \times C_A \times (RU_{max} - RU_1 - RU_2) - k_{d1} \times RU_1 - k_{a2} \times RU_1 + k_{d2} \times RU_2$$

$$dRU_2/dt = k_{a2} \times RU_1 - k_{d2} \times RU_2$$

where RU_1 and RU_2 are the response units for the first and second steps, respectively, C_A is the peptide concentration, RU_{max} is the maximal response unit (or equilibrium binding response), and k_{a1}, k_{d1}, k_{a2} and k_{d2} are the association and dissociation rate constants for the first and second steps, respectively. k_{a1} has units of 1/(M×sec), while k_{d1}, k_{a2} and k_{d2} have units of 1/sec, so that the total affinity constant for the whole process, K, has M^{-1} units (see legend to Table 3).

Biological activity assays

MIC against clinical indicator strains

For selected active peptides, MIC values were determined under more stringent conditions against a wider set of clinical, antibiotic-resistant bacterial indicator strains, and two C. albicans strains, using 96-well polypropylene plates (Life Technologies), pre-coated with BSA to reduce peptide binding [6]. The indicator strains used (listed in the caption to Figure 9) were grown to an optical density of 1.0 at 600 nm in half-concentrated MH broth, and diluted 1:10^5 with the same medium. 100 µl of this suspension were mixed with 100 µl of the appropriate increasing peptide dilution in successive wells and the MICs determined after 18 h of incubation at 37 °C. In these assays, only MIC values of 4 µM or less are reported, as they were considered to indicate a potent anti-microbial activity against a given indicator strain.

Bacterial membrane permeabilization kinetics

The permeabilization of the cytoplasmic membranes and outer membranes of *E. coli* by synthetic peptides was evaluated by following the unmasking of cytoplasmic β-galactosidase or periplasmic β-lactamase activity, using extracellular ONPG (Sigma, Saint Louis, U.S.A.) and CENTA (Calbiochem, Darmstadt, Germany), respectively, as substrates. For these experiments, the β-galactosidase constitutive, lactose-permease deficient ML-35 strain was used, exposed to a 5 µM peptide concentration [7,8]. Permeabilization of the cytoplasmic membrane of *S. aureus* was instead evaluated by following unmasking of cytoplasmic phospho-β-galactosidase activity, using the phosphotransferase deficient, 6-phospho-β-galactosidase constitutive 710A strain [8,9] following the same protocol, but using freshly prepared ONPG-6P [10] as substrate. As susceptibility to induced or spontaneous permeabilization, in this strain, appears to increase somewhat with time, freshly prepared bacteria were always used as soon as possible after resuspension in PBS.

Preparation of lymphocyte single cell suspensions

Lymphocytes were obtained from the spleens of healthy mice, mechanically disaggregated on sterile Petri dishes in 5 ml RPMI 1640 medium supplemented with 5 % (v/v) FBS. After filtering and centrifuging at 400***g*** for 5 min at 4 °C, erythrocytes were lysed by hypotonic shock, and following a further centrifugation, lymphocytes were re-suspended in PBS, counted by the Trypan blue exclusion test and diluted to the appropriate concentration with PBS.

Scanning electron microscopy

Lymphocyte suspensions, after challenge with the test peptides as described above, were re-suspended in PBS and diluted to 2×10^7 cell/ml. A drop of each cell suspension was layered onto slides previously coated with poly-L-lysine solution (0.1 mg/ml, Sigma), and allowed to adhere for two hours. Cells were then fixed with 2.5 % (w/v) glutaraldehyde in 0.1 M cacodylate buffer overnight. All procedures were at 4 °C. Samples were dehydrated in graded ethanol, vacuum dried and mounted onto aluminium SEM mounts. After sputter coating with gold, they were submitted for analysis with a Leica Stereoscan 430i instrument.

Figure captions:

Figure S1 CD spectra of selected peptides A) spectra in 5 mM SPB, pH 7.4; **B)** spectra in 10 mM SDS, 5 mM SPB; **P1** (·····); **P6** (**----**); **P3** (**—**); **P7** (-···-··-); **P10** (-·-·-).

Figure S2 Kinetics of bacterial membrane permeabilization by model peptides
Permeabilization of **A)** the outer membrane of *E. coli* ML-35 determined as unmasking of CENTA to periplasmic β-lactamase; **B)** the cytoplasmic membrane of *E. coli* ML-35 determined as unmasking of ONPG to cytoplasmic β-galactosidase and **C)** the cytoplasmic membrane of *S. aureus* 710A determined as unmasking of ONPG-6P to cytoplasmic phospho-β-galactosidase. Bacteria (10^7 CFU/ml in PBS) were exposed to 5 μM of: **P1** (—); **P2** (---); **P3** (—); **P4** (**···**); **P5** (—); **P6** (**-··**); **P7** (**-···**); **P8** (**-··**); **P9** (---); **P10** (**-···**); **P11** (**···**).

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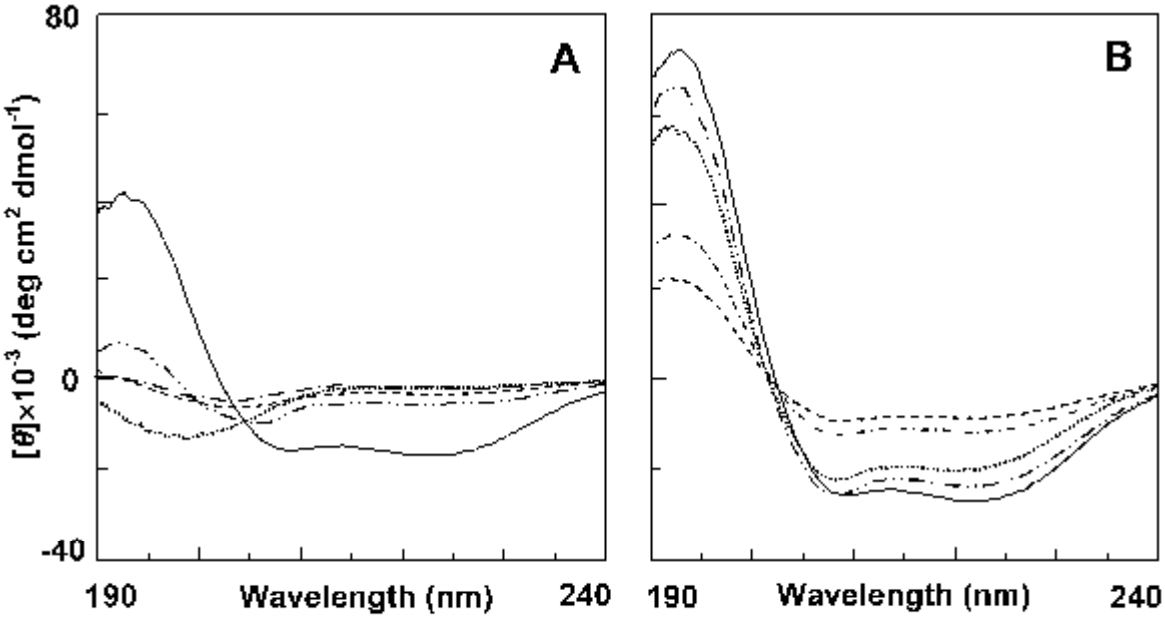
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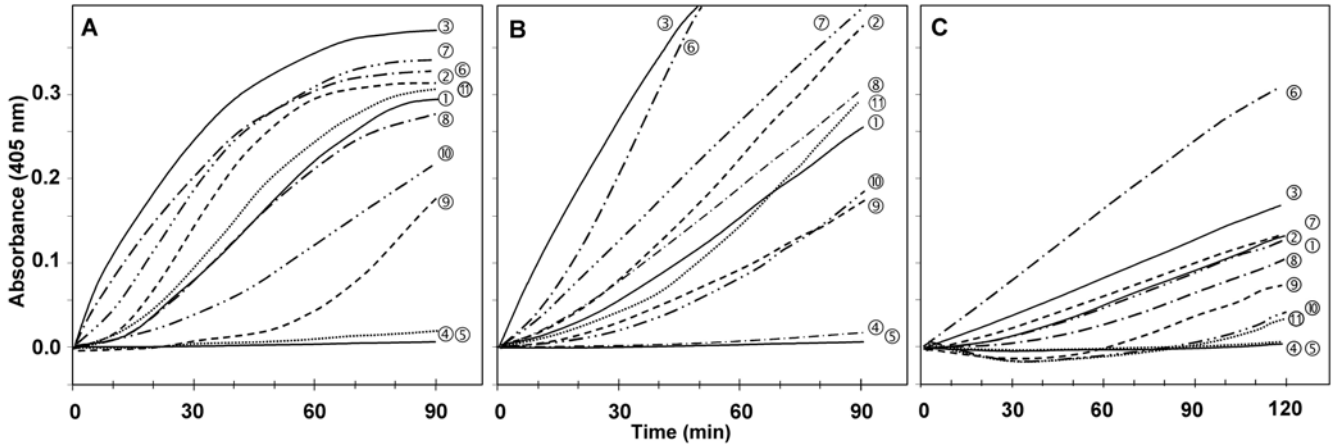
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