High-Throughput Discovery of Peptide Antibiotics: A Delicate Balance Between Antimicrobial Potency, Selectivity and Target Selectivity

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Antimicrobial peptides (AMPs) are key components of the innate immune systems of many organisms. AMPs function by permeabilizing microbial membranes, giving them an important advantage over conventional antibiotics as they may elude the selection of drug-resistance. Therefore, there has been increased interest in engineering AMPs and improving their biactivity over the last three decades. Yet the lack of obvious structure-function relationships or molecular design principles has obstructed the development of new AMPs. To circumvent this roadblock, we are developing a high-throughput approach to select AMPs that are optimized in all of the critical factors simultaneously. Previously in our lab, we identified a group of broad-spectrum antimicrobial peptides from a synthetic peptide library. Here, we use a degenerate library of the broad-spectrum AMPs. *ARVA (RGWALRVLV),* to study the potency, selectivity and mechanism of action of AMPs in the presence of concentrated human erythrocytes (10^7 cells/ml) which mimics the in vivo milieu. *ARVA,* and other AMPs, lose antimicrobial activity in concentrated erythrocytes. We developed a method to make direct measurements of peptide binding to cells which showed that loss of activity is due to weak host cell binding; coupled with the large mass excess of host cell vs. bacterial cells under physiological conditions. To identify AMPs with clinically-relevant activity, we are developing a novel, orthogonal high-throughput screen in which we select simultaneously for 1) peptide solubility; 2) lack of host cell lysis; 3) sterilization of a Gram positive microbe in the presence of concentrated erythrocytes, and 4) sterilization of a Gram negative microbe in the presence of concentrated erythrocytes. Our results show that rational library design and high-throughput screening is a promising approach to identify AMPs that have the needed balance between antimicrobial potency, solubility and target cell selectivity.

Human Lactoferrin Derivatives as New Targeted Weapons in Cancer Therapy

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In our study derivatives of a short cationic peptide derived from the human host defense peptide Lactoferricin (LFcin) were optimized in their activity and selectivity towards cancer cells. The negatively charged membrane lipid phosphatidylserine (PS) serves as a target for these peptides since PS is specifically exposed by cancer cells (1). The calorimetric and permeability studies showed that N-acetylation and even more the repeat sequences of derivatives of hLFcin leads to strongly improved interactions with the cancer mimic PS, whereas the healthy mimic phosphatidylcholine (PC) is only slightly affected by the lipopeptide. Tryptophan fluorescence of selective peptides revealed peptide penetration only into the PS membrane interface and circular dichroism showed change of structure by increase of amount of β-sheets just in the presence of the cancer mimic. We demonstrated that hLFcin derivatives also exhibit anticancer activity in vitro against several cancer cell lines, correlating with selective activity against the cancer model PS. Apoptosis and selective peptides induced apoptosis only in cancer cells, whereas melanocytes and fibroblasts remained unaffected at same concentrations, yielding specificity for cancer cells higher than 100-fold for some peptides. Currently, first in vivo studies are under progress.

The data indicate the need of high affinity to the target PS, a minimum length of 100 residues, such as "trefoil" domain was lost. The toxicity of toxins was decreased by increasing incubation time for heat treatment. The tolaasin of P. tolaasii, secretes a lipodepsipeptide toxin, tolaasin. It is a pore-forming peptide toxin and causes the disease by making pores on the membranes and disrupting the cellular and fruiting body structure of mushrooms. Forty-three bacteria were isolated from the cultivated mushrooms tissues of the farms reported the outbreaks of brown blotch disease and identified by the sequence comparison of 16S rDNA genes. Five different species of Pseudomonas, P1-P5 subgroups, were identified. The P1 group, including 43 strains, have been identified as the main pathogen secreting tolaasin peptide. P2-P5 groups are other than P. tolaasii and they also cause brown blotches in pitting and cultivation tests of oyster mushroom. In the pitting test using mushroom caps, all five subtypes were able to form brown blotches. These results suggest that subgroups P2-P5 also secrete tolaasin-like peptides causing brown blotches. In order to characterize varied pore-forming properties of these peptides, the effects of temperature on the toxicities of these peptide toxins were measured. The hemolytic activities of toxins from P1 to P5 subgroups were decreased by increasing temperature from 40 to 100°C. The toxicity of toxins was decreased by increasing incubation time for heat treatment. The tolaasin of P. tolaasii, P1 group, is an 18 amino acid-peptide, its molecular mass is 1985 Da, and it forms a left-handed α-helix. Characteristics of the peptide toxins of P2-P5 subgroups are not known. We have isolated these toxins and compared them with tolaasin by gel permeation chromatography, ion-exchange column chromatography, HPLC analysis, and mass spectrometry.

Cytoxicities of Various Pore-Forming Toxins Obtained from Strains of Pseudomonas Toluasi

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Pseudomonas tolaasi causes brown blotch disease on the cultivated mushroom rooms, such as Agaricus bisporus and Pleurotus ostreatus. It secretes a peptide...
toxin, tolasin. The toxin consists of 18 amino acids and its molecular mass is 1,985 Da. The mechanism of membrane-formation of tolasin molecule has not known in detail. However, tolasin channels had been demonstrated in the artificial lipid bilayer. In order to characterize the pathogenic properties of toxin peptides, we isolated 23 strains of P. tolaasi from the infected mush-rooms. To make sure that they are closely related but not the same bacteria, their 16S rRNA genes were sequenced and analyzed. They were divided into three subtypes, P1α, P1β, and P1γ, depending on the sequence of 16S rRNA genes, and 6, 16, and 1 strains were included in each subtypes, respectively. In the pitting test, all three subtypes were able to cause brown blotches on the surface of mushroom caps. Two subtypes, P1α and P1γ, showed hemolytic activities on erythrocytes; however, strains of P1β subtype did not show any hemolytic activity. Only three strains of P1α subtypes were able to form white lines with P. reactans, suggesting that the structures of their toxins are very similar to tolasin. Since cytotoxic effects of toxins obtained from the same subtype strains were slightly different, further analyses were performed by measuring metabolic activities of these bacteria with API kit. In detail, there were 5 patterns in general microbial characters, 7 in biochemical tests, 4 in assimilation tests, and 5 in fermentation tests. Strains of P. tolaasi were divided into 6 groups by API test. Peptide toxins were obtained from each subtypes and purified by gel permeation chromatography, ion exchange chromatography, and HPLC. Their structures and functions are under investigation.

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Interaction of an Antitumor Peptide with Lipids of the Cancer Plasma Membrane - Formation of Membrane Domains and Influence of Choles-terol
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R-DIM-P-LF11-322, derived from the cationic human host defense peptide lactoferricin, exhibits selective antitumor activity against several human cancer cell types. We have shown that cancer cells expose the negatively charged phosphatidylserine (PS) on the outer leaflet of the plasma membrane (1), which in healthy cells only comprises neutral lipids as phosphatidylcholine (PC) or cholesterol. Therefore the membrane interaction of R-DIM-P-LF11-322 with membrane mimics composed of mixtures of PS, PC and cholesterol was stud-ied. Lipid analysis of normal melanocytes and melanoma revealed a slight in-crease of PC and cholesterol in the cancer lipid extracts. Tryptophan fluorescence showed that the peptide penetrated only in the cancer mimics containing ratios of PS. Addition of cholesterol slightly reduced the af-finity of the peptide. Calorimetric studies with peptide and mixed liposomes of PS and PC indicated complete perturbation of the PS fraction leaving PC unaffected. Interestingly upon addition of cholesterol the peptide induced separation into PC and PS en-riched domains, but seemed to be slightly reduced in its effect. Studies using giant unilamellar vesicles showed that the peptide interacts on cancer model membranes slightly stronger in the absence of cholesterol for for-mation of extremely deformed and destroyed vesicles. In the presence of cholesterol the peptide however still induced shrinking of the vesicles and for-mation of many small PS domains. In summary the antitumor peptide strongly interacts only with its target PS on cancer cells. However, cholesterol seems to play a role in the modulation of membrane domain formation and reducing the membrane affinity, which may represent a mechanism for cancer cells to protect themselves against anti-tumor peptides.


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Pore Formation and Dynamics of Cytoxin-A on Supported Lipid Bilayer Membranes
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Observing the structure and dynamics of large protein assemblies on mem-branes has implications on understanding the general principles behind protein aggregation and engineering drug therapies. Dynamic and structural data on mesoscopic length and time scales are scarce for these systems. Cytoxin-A (CrA) is a pore forming toxin that is known to cause unregulated pores on various cell membranes. In order to study pore formation of CrA on model membranes, supported lipid bilayers (SLBs) of DMPG were incubated with varying concentrations of CrA. Pore densities were quantified using atomic force microscopy (AFM) and led to the observation that a threshold toxin con-centration is necessary for pore formation. The influence of the bilayer prep-aration protocol was observed by contrasting the pore densities on SLBs prepared using Langmuir-Blodgett (LB) deposition as well as vesicle deposi-tion. Pore formation at varying deposition pressures on different substrates (mica, silicon) were investigated using LB prepared bilayers. Interestingly, at a high toxin concentration of 8.82 nM (large pore density regime), membrane undulations were prominently observed in the AFM images. Understanding the lipid dynamics around protein assemblies has implications on protein signal-ling, stability and function. Fluorescence imaging using FRAP and FCS on SLBs incubated with CrA, and complementary molecular dynamics simul-a-tions were carried out to investigate the inherent heterogeneity in lipid dy-namics in the vicinity of the CrA pore.

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Solid State NMR Structure-Function Analysis of the Stress-Response Peptide TisB
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The 29-residue peptide TisB is expressed in E.coli in response to stress and has been suggested to break down the transmembrane potential by equilibrating the proton gradient [1,2]. We characterized its membrane bound structure using solid-state NMR and oriented CD (OCD). For the NMR analysis we individually solubilized several hydrophobic amino acids in TisB with CF3-L-Bpg, and/or with a 15N-label. CD analysis showed that all analogues remain z-helical, similar to the wild type peptide. 19F-NMR showed that TisB assumes a trans-membrane orientation, with its helix aligned parallel to the membrane normal, as confirmed by 15N-NMR and OCD. For a functional characterization we used a vesicle based fluorescence assay to test the breakdown of the pH gradient. The rate of pH equilibration as a function of peptide concentration revealed a Hill coefficient of 2, which is suggestive of dimer formation. This gave rise to the idea that an antiparallel dimer could be formed and stabilized in the membrane by four intermolecular salt bridges and one central H-bond at Gin19. To test this hypothesis, we synthesized several TisB mutants in which the charged res-idues engaged in the postulated salt bridges were mutually exchanged, and/or with a 15N-label. CD analysis showed that all analogues remain z-helical, similar to the wild type peptide.


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Phlp-Fire: A High-Contrast, Insertion-Triggered Fluorescent Probe for Targeting Tumors In Vivo
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pHLIP-FIRE (Fluorescence Insertion Reporter) is a novel diagnostic tool based on pHLIP (pH Low Insertion Peptide), a water soluble membrane peptide that inserts with membranes by forming a transmembrane helix at low pH. The fluorescence of pHLIP-FIRE is activated by separation of mutually quenching or donor-quencher fluorescent groups via the cleavage of a disulfide-linker when the C-terminus moves into the highly reducing environment inside the cell. Targeting studies in mice bearing breast tumors showed strong targeting of pHLIP-FIRE in vivo, giving a contrast index of 20 with respect to back-ground. This contrast is a significant enhancement in comparison with tradi-tional fluorescent pHLIP constructs. Experiments in vitro and in cultured cells show that the time-course of dequenching in pHLIP-FIRE is pH-dependent and quite slow (~ 1 - 2 days). Direct imaging with pHLIP-FIRE gives the first demonstration of pHLIP insertion and cargo translocation in...