Elastic and Structural Interactions of Engineered Cationic Antimicrobial Peptides (eCAPs) **WLBU2** and **D8** with Bacterial Lipid Model Membranes

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Thanks to Subra and John Katsaras for inviting me to talk at this interesting session at the ACS meeting. Although I have been a member in the American Chemical Society on and off for many years, I have never attended an ACS meeting, so this was a special occasion for me. Apparently I will have to wait a little longer to actually attend an ACS meeting. The title of my talk is (read). I am going to use the word Mimic in this to represent the lipid model membranes.

WLBU2: RR WVRR VRR WVRR VVR VVRR WVRR

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Charge: +13
Hydrophobic moment: $\mu H = 0.8$
Hydrophobicity: $\Delta G_{wif} = +5.54$
Length: 24 AAs
Types of AAs: 3
Secondary structure: lipid-dependent

Peter Di, PhD
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Peptide	Bacterial Killing Efficiency	
	PA01 MIC (μM) G(-) bacterium	MRSA MIC (μM) G(+) bacterium
WLBU2	3.5 ± 1 (N=4)	3.2 ± 1.1 (N=5)
D8	3 ± 1.2 (N=4)	3.2 ± 1.1 (N=5)

D8: RR WVRR VRR WVRR VVR VVRR WVRR

Given the problem of bacterial resistance to known antibiotics, one solution is to develop antimicrobial peptides. Since they primarily attack the cell membrane, resistance is slower to develop than to traditional antibiotics. These are my collaborators at the University of Pittsburgh, Drs. Toni Deslouches and Peter Di. Toni designs the peptides, taking into consideration: charge, hydrophobic moment, hydrophobicity, length and types of amino acids. This is called rational design. Peter Di made a small modification in WLBU2 by introducing the D-enantiomer of valine. He replaced 4, 6 or 8 valines and found that D8 was the most efficacious at killing bacteria and the least toxic. The inclusion of D-enantiomers prolongs the life of the peptide in vivo. These peptides are highly cationic with a charge of +13 and there are only 3 types of amino acids. Both WLBU2 and D8 are both very efficient at killing both G(-) and G(+) bacteria. (point to table and talk about MICs). The therapeutic index of these peptides is about 3.

LBU = lytic base unit (12AA's). They added W to increase activity in physiological salt conc.

In 2019 I published this paper in Soft Matter. It describes the elastic response of lipid model membranes to WLBU2 and D8. This paper is a precursor to the structural results that I will show you today. Akari Kumagai, a Physics undergrad student, is the first author on this paper since she contributed to the data collection at CHESS and also to the data analysis. In addition to postdocs and faculty colleagues, undergraduates contribute significantly to my research.

RESEARCHARTICLE

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Synergistic Biophysical Techniques Reveal Structural Mechanisms of Engineered Cationic Antimicrobial Peptides in Lipid Model Membranes

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Structural techniques used:

1. X-ray diffuse scattering (XDS)
2. Neutron reflectometry (NR)
3. Circular dichroism (CD)
4. Nuclear magnetic resonance (NMR)
5. Differential scanning calorimetry (DSC)
6. Molecular dynamics simulation (MD)

Mimics used:

1. Gram-negative inner membrane (G(-) IM)
2. Gram-positive cell membrane (G(+))
3. G(-) outer membrane (OM): KDO2
4. G(-) OM: Lipopolysaccharide (LPS) model
5. Typical eukaryotic membrane (Euk23)
6. Red blood cell membrane (Euk50)

The structural work involved many investigators and was truly an international collaboration with one researcher from Argentina, one from India, and two from Germany. Frank Heinrich played a dominant role in the work supplying data on peptide locations in membranes using neutron reflectivity. There were six biophysical methods used and six different lipid model membranes. This work was a Herculean effort which took us four years to complete. Mention the methods used and that there were six lipid model membranes.

These are the three mimics of the outer membrane of Gm negative bacteria that we used: Lipid A (point), KDO2 (point) and a mixture of LPS (which is this entire lipid, consisting of Lipid A and then the core oligosaccharides and finally the O-antigen, which can have many repeats of these final sugar residues. We took LPS from Sigma and mixed it with in a 1:3 mole ratio with DLPG, which has the similar chain length as LPS. This allowed the LPS to fluctuate and then we were able to use diffuse X-ray scattering results. KDO2 = Two 3-deoxy- α -D-manno-oct-2-ulosonic acid (α -KDO) residues.

These are the data collected at CHESS using x-ray diffuse scattering (XDS). From one sample we obtain both elasticity and structural results from the LAXS and lipid chain order results from the WAXS. So one sample can tell us how stiff or soft the membrane is, how thick it is, what is the area/lipid and are the chains ordered or disordered. This saves an enormous amount of time – differential hydration levels are now required. This particular sample was G(-) inner membrane with WLBU2 at a 250:1 molar ratio.

Slide 7

Elasticity and Chain Order

First I will talk about elasticity and chain order.

Here you see some results from the Soft Matter paper. For G(-) IM there was first an increase in stiffness at low concentration, and then a dramatic softening at higher concentration of both peptides. Similarly the lipid chains became more ordered and then more disordered as a function of concentration. This is what we call non-monotonic behavior. It also occurred, even more dramatically, in our previous publication in 2018 with colistin. At that time we suggested that domain formation is occurring with domains of different stiffness which would create domain walls between them. These walls would cause defects that would allow water and ions to flow out of the bacteria, thus killing them. We observed similar behavior for the G(+) mimic. Remember that both peptides kill G(+) and G(-) bacteria efficiently.

Here are some other elasticity and chain order results. For the LPS model there were only small changes in K_c with increasing concentration for WLBU2 and D8 and a decrease in chain order caused by both peptides.

For Euk 23, which is a mimic that is typical of many eukaryotic cells, both peptides behaved similarly with a general softening. This behavior was quite different from G(-) IM and G(+), in that the changes are monotonic rather than non-monotonic. Both peptides are toxic to leukocytes, which suggests that the softening could cause a micellization. But not domain walls.

In the red blood cell mimic that contained 50% cholesterol, there was a general softening (point) and a disordering of chains (point) when WLBU2 interacted with this membrane, like in Euk23. However for D8 there was a major membrane stiffening that did not revert to the control K_c value. This is a good example of how elasticity and toxicity are correlated: while WLBU2 is toxic to red blood cells, D8 is not. It is perhaps that the membrane stiffening caused by D8 protects the RBC cell from leakage. The lipid chains also became more ordered in the presence of D8.

Structure

OK, so what about the structure?

So we were hoping to see a structural correlation with the changes in elasticity. You might think that a softening, for example, would lead to a bilayer thinning, and a stiffening would lead to a bilayer thickening. However, we did not observe this correlation between elasticity and structural results for WLB2 or D8, while we had seen this correlation with the other AMP, colistin.

These data show that the area/lipid (black lines) increases with increasing WLB2 (solid symbols) or D8 (open symbols), while the bilayer thickness (red lines) decreases when the peptides are in the Gram-negative inner membrane (point) or gram-positive mimic (point) or in a typical eukaryotic membrane (point). There was little change for either peptide in the outer membrane models (point). In Euk50, which has 50 mole % cholesterol, like the RBC, there was actually a small decrease in area and increase in membrane thickness.

Here you see my research team in the summer of 2018 at the CHESS synchrotron.

I am fortunate to be able to collaborate with Frank Heinrich who works at NIST. Here you see some of the results that Frank obtained with mixtures of the different lipid model membranes with both peptides. While XDS can obtain the area/lipid and bilayer thickness very accurately, it is more difficult for x-ray scattering to also obtain the precise location of the peptide in the membrane, due to the small difference in electron density of peptides and lipids. By changing the solvent composition from H₂O to D₂O, large differences in contrast in the headgroup region can be seen with neutron reflectivity, which allows determination of peptide location. In these experiments, we embedded the peptides with the lipids, sonicated them into salt-laden vesicles and used osmotic bursting to deposit a single tethered bilayer onto a silicon wafer. We had previously compared this method to adding the peptide through the aqueous phase, and found the same peptide location. As you can see there is a dual location of both WLBUE2 and D8 in both G(-) and G(+) membranes, which indicates that some of the peptides remain in the headgroup region, while others are drawn to the hydrophobic interior. For Euk23, a normal eukaryotic cell containing 23 mole % cholesterol, both WLBUE2 and D8 are located directly underneath the headgroup in the so-called interfacial region. That is not the case in Euk50, which is a red blood cell mimic, where both peptides are located in the headgroup region, but WLBUE2 in slightly more interior. For LPS model both peptides are deep into the hydrocarbon region. In KDO2, both peptides are primarily in the headgroup region, although the inner leaflet headgroup may have pulled them across.

The first thing that MD simulators ask me when I ask them to collaborate is, What is the secondary structure of the peptide in the membrane? Fortunately I have mentored several very enthusiastic undergrad students who collected data using the Jasco 715 instrument that is located in the chemistry department at CMU. Aria Salyapongse collected many CD scans in the fall of 2018 and she carried out the data analysis. Our main method to analyze the CD data is to use shape analysis. I.e., the four main shapes, alpha-helix, beta sheet, beta turn and random coil are fit using linear least squares to the CD data. I think that this is superior to determining secondary structure by comparing to data banks of large proteins, either in membranes or in aqueous phase, because these are small peptides and they are not influenced by parts of a larger protein. What you can see in this table is that in aqueous phase, WLBUE2 has very little helix, but is primarily random with some beta sheet. The R^2 value indicates the goodness of the fit. In the other mimics shown here, G(-) IM, KDO2, G(+) WLBUE2 was primarily helical with the rest mostly beta sheet. D8 could not be analyzed using the shape analysis, because the D-enantiomers actually have an inverted shape compared to L-enantiomer. When scanned in water there was only just this one broad peak that does not match any of the four motifs. It turns out that this peak is from tryptophan. So what to do? Fortunately, I was able to obtain NMR data with the lab of Dr Daniel Huster at the University of Leipzig.

So for secondary structure we turned to our collaborators at the University of Leipzig, Daniel Huster and his postdoc Anja Penk. For this they had to use expensive, ^{13}C labeled peptides for each type of amino acid. The technique is called HSQC-TOCSY (homonuclear single quantum coherence spectroscopy – total correlation spectroscopy). They found that D8 always aligned with a random coil chemical shift difference, while WLBU2 became helical in G(-) IM, Euk50 and TFE.

In addition to solving the secondary structure, they also carried out some other studies. What you can see here on the left is the phosphorous ^{31}P NMR spectra as each peptide was added to G(-) IM and Euk50 membranes. The results are shown for per-deuterated POPG, but they were the same for per-deuterated POPE, indicating a homogenous samples. These spectra indicate that the lipids are in the fluid, liquid crystalline phase. For additions of both WLBU2 and D8 to G(-)IM, the chemical shift anisotropy, which corresponds to the width of these peaks, increased. This indicates that in this mimic, both peptides either reduced the headgroup motion or altered the headgroup orientation. That was not the case when the peptides were added to Euk50, where the headgroup was not affected. However, you can see that there is a sharp isotropic peak at 0 ppm that indicates that a fraction of the lipids reorients isotropically with respect to the magnetic field on a fast time scale ($\ll 100$ msec). This isotropic is not seen in the control or in the D8 peptide sample, so it indicates a different interaction for WLBU2 compared to D8. In addition, they carried out static deuterium NMR, and showed that there is a reduction in lipid chain order when either peptide is added. They calculated that this would result in a membrane thinning of $\sim 1.6 \text{ \AA}$, very close to what we have determined for WLBU2 using XDS. Significantly they again observed a sharp component in the deuterium-NMR spectrum (point) only with WLBU2, not with D8 or the control. This indicates a fast reorientation of the lipids over a highly curved structure, such as a pore, or a micelle, which gave a good clue as to the different mechanisms of WLBU2 or D8 in the RBC mimic (remember that D8 is not toxic while WLBU2 is toxic to RBCs).

JC Gumbart and his postdoc Anna Pavlova at Georgia Tech were able to carry out MD simulations with Charmm36 for lipids and Charmm36m for protein. 200 nanoseconds without the protein and 400 nanoseconds with the protein. WLB2 moved from the aqueous phase to the lipid headgroup and did not enter more deeply into the bilayer, which agreed with the neutron reflectometry results. In the peptide red is R, yellow is V, blue is W. Small – only 72 lipids.

With all this information, what do we think is happening? Both peptides probably penetrate along the domain walls, although we are not calling this a pore. We think that there is a different mechanism for bacterial killing and cell toxicity.

People who did the work!

Tristram-Nagle group, summer, 2018

Tristram-Nagle group, summer, 2019

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THANKS FOR YOUR REMOTE ATTENTION!

Thanks for your attention.