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The effect of the placement and total charge of the basic amino acid clusters on antibacterial organism selectivity and potency

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ABSTRACT

Extensive circular dichroism, isothermal titration calorimetry and induced calcein leakage studies were conducted on a series of antimicrobial peptides (AMPs), with a varying number of Lys residues located at either the C-terminus or the N-terminus to gain insight into their effect on the mechanisms of binding with zwitterionic and anionic membrane model systems. Different CD spectra were observed for these AMPs in the presence of zwitterionic DPC and anionic SDS micelles indicating that they adopt different conformations on binding to the surfaces of zwitterionic and anionic membrane models. Different CD spectra were observed for these AMPs in the presence of zwitterionic POPC and anionic mixed 4:1 POPC/POPG LUVs and SUVs, indicating that they adopt very different conformations on interaction with these two types of LUVs and SUVs. In addition, ITC and calcein leakage data indicated that all the AMPs studied interact via very different mechanisms with anionic and zwitterionic LUVs. ITC data suggest these peptides interact primarily with the surface of zwitterionic LUVs while they insert into and form pores in anionic LUVs. CD studies indicated that these compounds adopt different conformations depending on the ratio of POPC to POPG lipids present in the liposome. There are detectable spectroscopic and thermodynamic differences between how each of these AMPs interacts with membranes, that is position and total charge density defines how these AMPs interact with specific membrane models and thus partially explain the resulting diversity of antibacterial activity of these compounds.

1. Introduction

The crisis is caused by the dramatic and continued evolution of drug resistant strains of bacteria[1,2] has stimulated an extensive world-wide research effort to develop new classes of compounds that exhibit novel mechanisms of antibacterial activity.[1,3–8] Natural and synthetic, antimicrobial peptides (AMP) offer promise as new therapeutic agents because of their novel mechanisms of antibiotic activity.[4,6,9–12] In our laboratory we developed a series of novel AMPs incorporating unnatural amino acids into the primary sequence of the peptide. These compounds have shown low μM to nM in vitro efficacy and selectivity against several bacteria strains including Gram positive, Gram negative and mycobacterium.[13,14] The major advantage of incorporating unnatural amino acids in the primary sequence of peptide therapeutics is the increased metabolic stability[11,15–18] thus increasing the therapeutic potential of peptide based drugs.

It is generally accepted that electrostatic interactions are the first step in the process of an AMPs binding to the surface of a cell membrane. All AMPs exhibit a high net positive charge[19] while most bacterial cell membranes contain a relatively high percentage of negatively charged phospholipids when compared to mammalian cell membranes.[20] The resulting difference in the electronic character of the cell membranes thus explains the inherent selectivity of AMPs for bacteria over mammalian membranes.[5]

It is well documented in the literature that the selectivity and potency of an AMP against a particular organism is defined in large measure by the complementary nature of the physicochemical surface properties of the cell's membrane and the interacting AMP.[5,6,8,21–24] The chemical composition of bacterial membranes

Abbreviations: Ahx, 6-aminohexanoic acid; AMP, antimicrobial peptide; βAla, beta alanine; CD, circular dichroism; Gaba, gamma aminobutyric acid; ITC, isothermal titration calorimetry; LUV, large unilamellar vesicles; MIC, minimum inhibitory concentration; MR, *Mycobacterium ranae*; Oic, octahydroindolecarboxylic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt); QSAR, quantitative structure–activity relationship; Tic, tetrahydroisoquinolinecarboxylic acid; SA, *Staphylococcus aureus*; ST, *Salmonella typhimurium*; SUV, small unilamellar

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varies greatly between different strains, therefore the interaction of a particular AMP will also vary between different strains of bacteria.[19,23,24] The hypothesis upon which our work is based is that the 3D-physicochemical surface properties of a cell's membrane (bacterial or mammalian) interact with those of the approaching AMP in a very specific way, thus defining the resulting organism selectivity and potency. These AMPs exhibited different in vitro activity against *Staphylococcus aureus* and *Mycobacterium ranae* bacteria which have chemically very different cell membranes. Therefore, we hypothesized that the differences in the observed biological activity was a direct manifestation of the different physicochemical interactions that occur between these peptides and the cell membranes of the *S. aureus* and *M. ranae* bacteria.[25] For this hypothesis to be correct different physicochemical descriptors must correlate with the anti-bacterial activity of these compounds against *S. aureus* and *M. ranae* bacteria. Two 3D-QSAR (quantitative structure–activity relationship) models defining the physicochemical properties of these AMPs required for activity against *S. aureus* and *M. ranae* bacteria were developed. It was determined that there are five physicochemical descriptors necessary to define the activity of these AMPs against *S. aureus* and that five different physicochemical descriptors are necessary to define the activity of these AMPs against *M. ranae*.[25] These results support the hypothesis that for any particular AMP, organism selectivity and potency are controlled by the chemical composition of the target cell membrane.

These AMPs were designed to systematically investigate the influence of small structural changes on the peptide's surface physicochemical properties, and then determine their effect on the binding to membrane model systems. As pointed out by several researchers in order to develop AMPs as potential therapeutic agents it is critical to develop an understanding of the basis for the different interactions that occur between the AMP and zwitterionic and anionic lipids in order to improve selectivity for bacterial cells.[11,26,27]

Micelles are excellent models to study the surface interactions that occur between peptides and membranes since micelles do not form bilayers and therefore, the peptides can't fully insert into the micelle to form pores as is the case with phospholipids. For this investigation, SDS micelles[28] were selected as a simple model for anionic lipids of prokaryotic cells and DPC micelles[29] as a simple model for zwitterionic lipids of eukaryotic cells. Large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were selected as a simple model for the zwitterionic membranes of mammalian cells and LUVs consisting of (4:1) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG) were selected as a simple model for the anionic membranes of bacterial cells to investigation the inserted state interaction associated with membrane binding.[30] The selection of mixed POPC/POPG lipids was not the best choice to mimic the lipid composition of bacteria membranes since POPC is normally not found in bacterial membranes, however it was selected to allow for comparison to previously reported investigations of AMP lipid interactions.[27,31–36] As pointed out by Huang and co-workers 'clarifying the pore forming mechanism' of a peptide 'will facilitate ... development of antimicrobial peptides as human therapeutics'.[37] This requires an understanding of the binding process to both zwitterionic and anionic membrane.

2. Materials and methods

Sodium dodecyl sulfate (SDS) and Bis–Tris buffer were purchased from Sigma–Aldrich. Monobasic and dibasic sodium phosphate, EDTA and NaCl were purchased from Fischer Scientific. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG) and dodecylphosphocholine (DPC) were purchased from Avanti Polar Lipids. High purity calcein was purchased from Invitrogen. All chemicals were used without further purification.

2.1. Peptide synthesis

Peptide synthesis was performed either manually using *t*BOC chemistry or with an automated peptide synthesizer using FMOC chemistry[38,39] as previously reported.[13,14,40] All peptides were purified by Reverse Phase HPLC using an Agilent 1100 Series Preparative Instrument using a Vydac C18 Reverse Phase Preparative HPLC Column as previously reported.[13,14,40] All purified peptides were analyzed again by HPLC and Mass-Spec. Mass Spectral analyses using a Finnigan LTQ ESI-MS instrument running Xcalibur 1.4SR-1 or a Kratos PC Axima CFR Plus instrument (MALDI) running Kompact V2.4.1. ESI-MS showed multiply charged ions and the accurate mass was calculated. MALDI analyses were performed in reflectron mode.[14,40]

2.2. Preparation of POPC and POPC/POPG SUVs

The appropriate amount of dry POPC or 4:1 POPC/POPG (mol to mol) was weight out to yield a final lipid concentration of 35 mM. The lipid was dissolved in chloroform and vortexed for 3 min. The sample was dried under nitrogen for 4 h and under high vacuum overnight. The lipid was then hydrated with 2 mL of buffer (40 mM sodium phosphate, pH 6.8) and vortexed extensively. SUVs were prepared by sonication of the milky lipid suspension using a titanium tip ultra-sonicator (Qsonica Sonicators model Q55) for approximately 40 min in an ice bath until the solution became transparent. The titanium debris were removed by centrifugation at 14,000 rev./min for 10 min using an Eppendorf table top centrifuge.[41] Final lipid concentration used for CD studies was 1.75 mM.

2.3. Preparation of POPC and POPC/POPG LUVs

A defined amount of dried POPC or 4:1 POPC/POPG (mol to mol) was weight out and suspended in buffer (40 mM sodium phosphate, pH 6.8) and spun for 30 min resulting in a total lipid concentration of 1.75 mM for CD studies and 35 mM for ITC studies. Other concentrations used are noted in the text. Large unilamellar vesicles (LUVs) were prepared by extrusion using a Mini-Extruder (Avanti Polar Lipid Inc.).[42,43] The solution was extruded through a 100 nm pore size polycarbonate membrane 21 times. After extrusion the LUVs were allowed to 'rest' for at least 2 h before use to allow equilibration to occur. The final lipid concentration was calculated based on the weight of the dried lipid.[31,33,40,41,44,45] Previously Kennedy and co-workers reported the preparation of LUVs using this procedure resulted in a homogeneous population of LUVs with >95% of the particles falling into the particle size range of 70–100 nm.[46] Kennedy and co-workers have also previously shown by ^{31}P NMR that these LUVs are unilamellar.[46]

2.4. LUVs for dye release experiments

A defined amount of dried POPC or 4:1 POPC/POPG LUVs (mol to mol) was weighed and suspended in a calcein-containing buffer (70 mM calcein, 10 mM Bis–Tris, 150 mM NaCl, 1 mM EDTA, pH 7.1, the pH was corrected using 3 mM NaOH and the final lipid concentration was calculated based on dilution). The resulting solution was vortexed for 1 min (5 times). The calcein-encapsulated LUVs were extruded using the same technique as above. Following extrusion the unencapsulated calcein was removed by gel filtration

on a Sephadex G50 column (eluent: buffer containing 10 mM Bis–Tris, 150 mM NaCl, 1 mM EDTA, pH 7.1). The fraction containing calcein-encapsulated liposomes was collected and retained for fluorescence studies.[31,33,40,41,44,45,47–49] Prior to use each batch of calcein-encapsulated LUVs were subjected to a self-quenching efficiency test. The self-quenching efficiency (Q) for each lipid suspension was set a minimum value of 80% before it could be used in these investigations. The Q value was calculated using the following equation: $Q = (1 - (F_0/F_T)) \times 100\%$ where F_0 and F_T are the background fluorescence of the lipid suspension and the total fluorescence after the addition of a solution of 10% Triton X, respectively.[27,29,50,51]

2.5. Circular dichroism

Peptide solutions of 75 μM dissolved in 40 mM phosphate buffer (pH 6.8) were used in these investigations. Binding studies were conducted using SUV and LUV preparations consisting of 1.75 mM POPC or4:1 POPC/POPG in 40 mM phosphate buffer (pH 6.8) with peptide concentrations of 100 μM. For the binding studies in the presence of micelles, 100 μM peptide was used with either 80 mM DPC or SDS in 40 mM phosphate buffer (pH 6.8). All CD spectra were obtained by acquiring 8 scans on a JASCO J-815 CD Spectrometer using a 0.1 mm cylindrical quartz cell (Starna Cells, Atascadero, CA) from 260 to 195 nm at 20 nm/min, 1 nm bandwidth, data pitch 0.2 nm, response time 2.0 s and 5 mdeg sensitivity at room temperature (~25 °C). Contributions due to micelles and LUVs and SUVs were eliminated by subtracting the lipid spectra of the corresponding peptide-free solutions. All analysis of CD spectra was conducted after smoothing (with a means-movement function) and conversion to molar ellipticity using the JASCO Spectra Analysis program.[30,40,42] CD spectra that exhibited HT values of greater than 400 were not used due to excessive light scattering and/or absorption. Titration studies were conducted using concentrations of POPC or 4:1 POPC/POPG LUVs varying from 0.8 to 12 mM.

2.6. Isothermal titration calorimetry

Data was acquired using a Microcal VP-ITC calorimeter (Microcal, Northampton, MA). All experiments were run in 40 mM sodium phosphate buffer, pH 6.8, at 25 °C. All solutions were degassed for approximately 10 min under vacuum before loading the reaction cell and syringe. For full titration experiments, 15 μL aliquots of 35 mM lipid solutions in buffer were titrated into 1.4 μL of peptide (100–200 μM). For binding single injection experiments, 15 μL of dilute samples of peptide (100–200 μM) were titrated into excess lipid (15–20 mM). A stirring speed of 220 rpm and injection duration of 30 s were chosen to ensure sufficient mixing while keeping the baseline noise to a minimum. To ensure complete equilibration between injections, a delay of 700 sec between injections was used. The background heat of dilution was obtained by titrating LUVs into the reaction cell containing only buffer and was subtracted prior to analysis. Data was analyzed with Origin® software (version 7.0). ITC data collection was obtained in duplicate in an effort to ensure reproducibility.[31,33,40–42,44,45]

There are two different models to consider when extracting thermodynamic data from ITC thermograms. The first and much simpler model, does not take into account the electrostatic interactions between the peptide and the membrane surface while the second considers these interactions.[44] The former model, and simplest of the two, involves the partitioning of the peptide between the aqueous phase and the lipid membrane, assuming (Eq. 1) as determined by Wieprecht and Seelig[44] is a linear relationship:

$$X_b = K_p c_f \quad (1)$$

where X_b is the molar ratio of membrane-bound peptide per lipid, c_f is the concentration of free peptide in solution and K_p is the partition coefficient.

The second model takes into consideration electrostatic interactions. Given that the peptides under investigation are highly positively charged and the LUVs surface contains regions of positive and negative charge although the net charge of the POPC LUVs is neutral, the second model is the more relevant for our analysis. The membrane surface contains localized regions of negative charge therefore attracting the positively charged residues of the peptide. This results in an increase of peptide concentration at the surface of the membrane, c_m in comparison to the bulk solution, c_f.[52] Therefore, (Eq. 2), [34,44,53,54] is more appropriate for the analysis of our data.

$$X_b = K c_m \quad (2)$$

By plotting X_b versus c_m, K can finally be obtained as it is the slope of the line.

Knowing K allows for the calculation of other thermodynamic data, including free energy (ΔG) and entropy (ΔS) from the following (Eqs. 3 and 4). [34,44,53,54]

$$\Delta G^0 = -RT \ln 55.3K \quad (3)$$

$$\Delta G^0 = H - \Delta TS \quad (4)$$

2.7. Calcein leakage assays

Peptide induced calcein leakage was investigated using an ISS PC1 photon counting spectrofluorometer (ILC Technology) at an excitation wavelength of 494 nm and an emission wavelength of 518 nm. An aliquot of peptide (10–50 μL) in buffer (10 mM Bis–Tris, 150 mM NaCl, 1 mM EDTA, pH 7.2) was added to the cell containing LUVs (36.6 μM lipid concentration) to give an overall peptide concentration of 4–20 μM. The fluorescence intensity was measured every minute for the first 20 min of the experiment and then every 10 min until the emission intensity showed no further significant increase (approximately 90 min). One hundred percent leakage was determined with the addition of 50 μL of 10% (v/v) Triton X in distilled water. The apparent percent leakage was calculated using the following equation: % leakage = $[(F - F_0)/(F_T - F_0))] \times 100\%$, where F_0 and F_T are the initial fluorescence before introduction of peptide and after the addition of Triton X, respectively.[33,40,42,47–49]

Table 1
Amino acid sequences of the peptides used in this investigation

Compound	Amino acid sequence
23	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKKK-$CONH_2$
39	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKKKK-$CONH_2$
64	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKK-$CONH_2$
61	Ac-**KKKK**-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-$CONH_2$

Table 2
In vitro activity, MIC activity (μM), against common bacterial strains[1]

Compound	*Salmonella typhimurium* (μM)	*Staphylococcus aureus* ME/GM/TC resistant (μM)	*Mycobacterium ranae* (μM)	*Bacillus subtillis* (μM)
23	10	3	10	1
39	10	3	3	1
61	100	30	3	Not tested

Table 3
In vitro activity, MIC activity (μM), against Gram negative and other drug resistant bacteria strains[2]

Compound	23	64	61
Acinetobacter baumanii ATCC 19606	3.2	6.7	12.8
Acinetobacter baumanii WRAIR	3.2	3.4	12.8
Staphylococcus aureus ATCC 33591	205	54	102
Yersinia pestis CO92	205	27	13.1
Brucella melitensis 16 M	205	54	51
Brucella abortus 2308	205	27	51
Brucella suis 23444	205	>216	25.6
Bacillus anthracis AMES	12.8	27	0.4
Francisella tularensis SCHUS4	100	0.02	51
Burkholderia mallei	205	0.02	204
Burkholderia pseudomallei	205	0.02	204

3. Results and discussion

LUVs or SUVs comprised of POPC were selected as a simple zwitterionic bilayer models whereas LUVs or SUVs consisting of 4:1 POPC/POPG were selected as a simple anionic bilayer models.[30] LUVs were selected instead of SUVs for calcein leakage and ITC studies because they are considered to be more closely related to biological membranes since LUVs are not subject to the increased curvature stress associated with SUVs,[41,55] which as a result decrease the stability of SUVs.[55–57] Understanding what physicochemical properties differentiate between binding with zwitterionic and anionic LUVs is critical in the development of these compounds as organism selective antibacterial drugs.[11]

As previously stated it is well documented in the literature that the net positive charge plays a critical role in the initial attraction of the AMPs to the surface of the target cell's membrane.[58–61] If the net positive charge is too low, the attractive electrostatic interaction between the peptide and the membrane may be insufficient to attract the peptide to the surface of the membrane. If the net positive charge is too high, the repulsive electrostatic interactions between incoming peptides and peptides already bound to the surface of the membrane may dominate, thus repelling additional peptides away from the surface of the membrane preventing the AMPs from reaching the necessary critical concentration on the surface required for pore formation to occur. If the positive charge is dispersed over the peptide backbone as is the case with melittin and pardaxin, the AMP may exhibit poor organism selectivity and be equally toxic to mammalian and bacterial cells.[11] This investigation focuses on the number of positively charged residues clustered at either C- or the N- terminus of the peptide sequence and the role this plays in organism selectivity and potency. For this investigation we have focused on four compounds with very interesting biological activity.

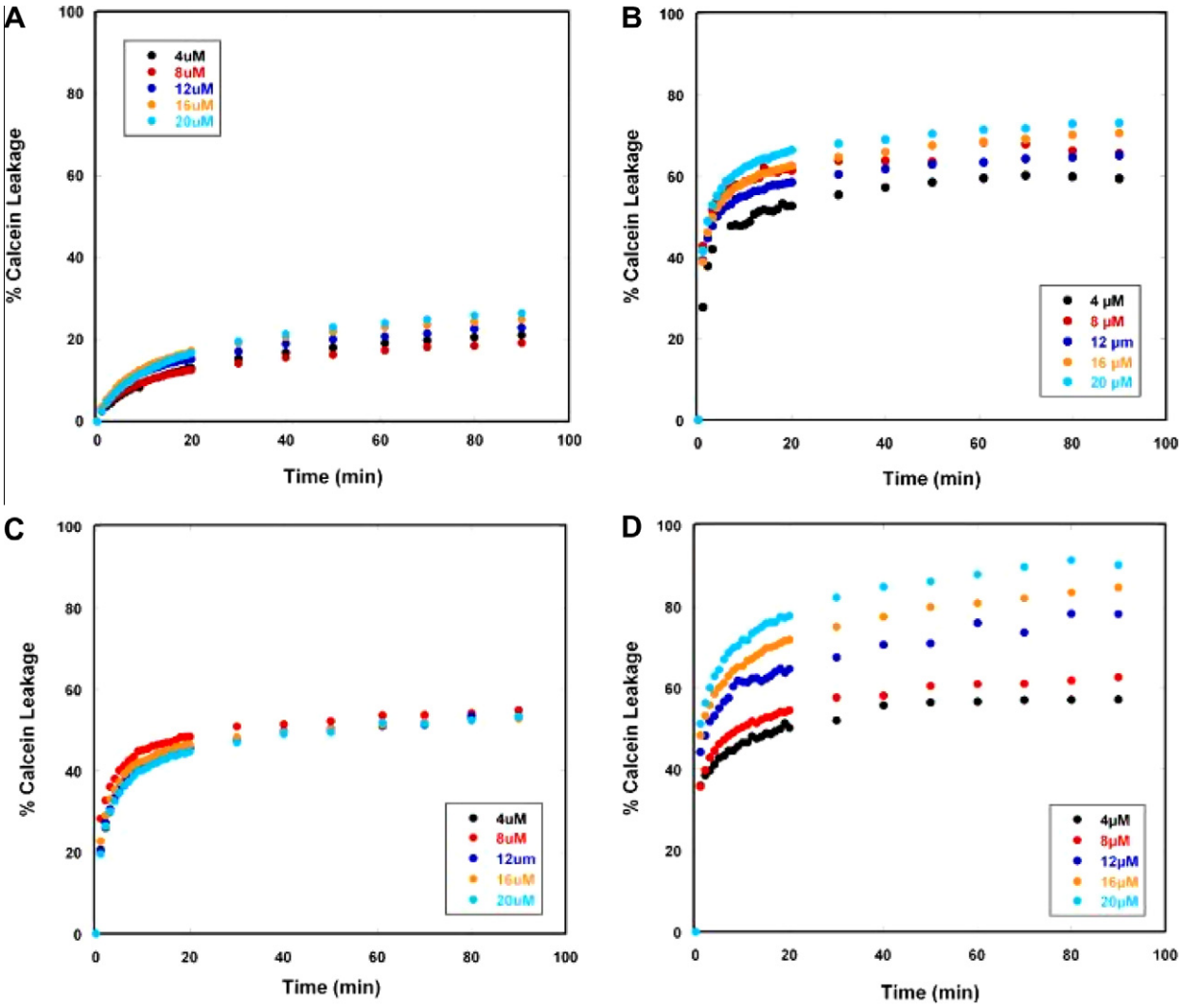

Figure 1. The time dependent release of calcein from POPC LUVs induced by increasing concentrations of compounds **23** (A), **64** (B), **39** (C), and **61** (D) as measured by fluorescence.[30–32] The unit of time is minutes.

Compound **23** has four Lys residues at the C-terminus, this number is decreased to three in compound **64** and increased to five for compound **39**. The cluster of four Lys residues is relocated to the N-terminus in compound **61**. The amino acid sequences for the AMPs used in this investigation are given in Table 1.

The in vitro antibacterial activity of these compounds clearly documents the critical role played by the placement and number of positively charged residues in defining antibacterial activity. The in vitro activity of compounds **23** (four Lys residues at the C-terminus), **39** (five Lys residues at the C-terminus and **61** (four Lys residues at the N-terminus) against common bacterial strains (Table 2), are very similar. Overall, each compound exhibited low μM, MIC values and their activity is very similar with the exception of the decreased activity of compound **61** against *Salmonella typhimurium*. However, in the presence of several Gram negative and Gram positive bacteria strains (Table 3), these compounds exhibited a range of activity for compounds **23** (four Lys residues at the C-terminus), **64** (three Lys residues at the C-terminus) and **61** (four Lys residues at the N-terminus). Compound **23** is the least active of the series except in the case of *Acinetobacter baumanii*. Either compounds **64** or **61**, if not both, have increased activity against each bacteria strain investigated as compared to compound **23**. Unfortunately, antibacterial activity against select agents is not available for compound **39**, so any conclusions made will be based on its activity with the common bacterial strains. The observed differences in activity against the various strains of bacteria are most likely a result of the differing percentages of the phospholipids found in the cell membranes. For example the Gram positive strains *staphylococcus aureus* 57% of the lipid composition is POPG, while *staphylococcus epidermidis* 90% of the lipid composition is POPG, and for *Bacillus subtilus* the percentage of POPG is reduced to 29%.[62] While the Gram negative strains *Salmonella typimurium*, *Pesudomonas cepacia* and *Escherichia coli* contain only 33, 18 and 6 percent POPG respectively.[62]

3.1. Calcein leakage studies

It is well documented that these AMPs exhibit antibacterial activity,[13,14] and they were designed to be members of the mechanistic class of AMPs known as membrane-disruptors.[21,52,63] However the mechanisms of action of these AMPs has not yet been determined previous to this investigation. Peptide induced calcein leakage from LUVs monitored through fluorescence is a well-documented technique for probing AMP activity to confirm membrane disruption.[35,64] Different concentrations of peptide (4–20 μM) were introduced to solutions of either POPC or 4:1 POPC/POPG LUVs and their induced fluorescence due to leakage of calcein was monitored over a 90 min time period. As can be seen in Figure

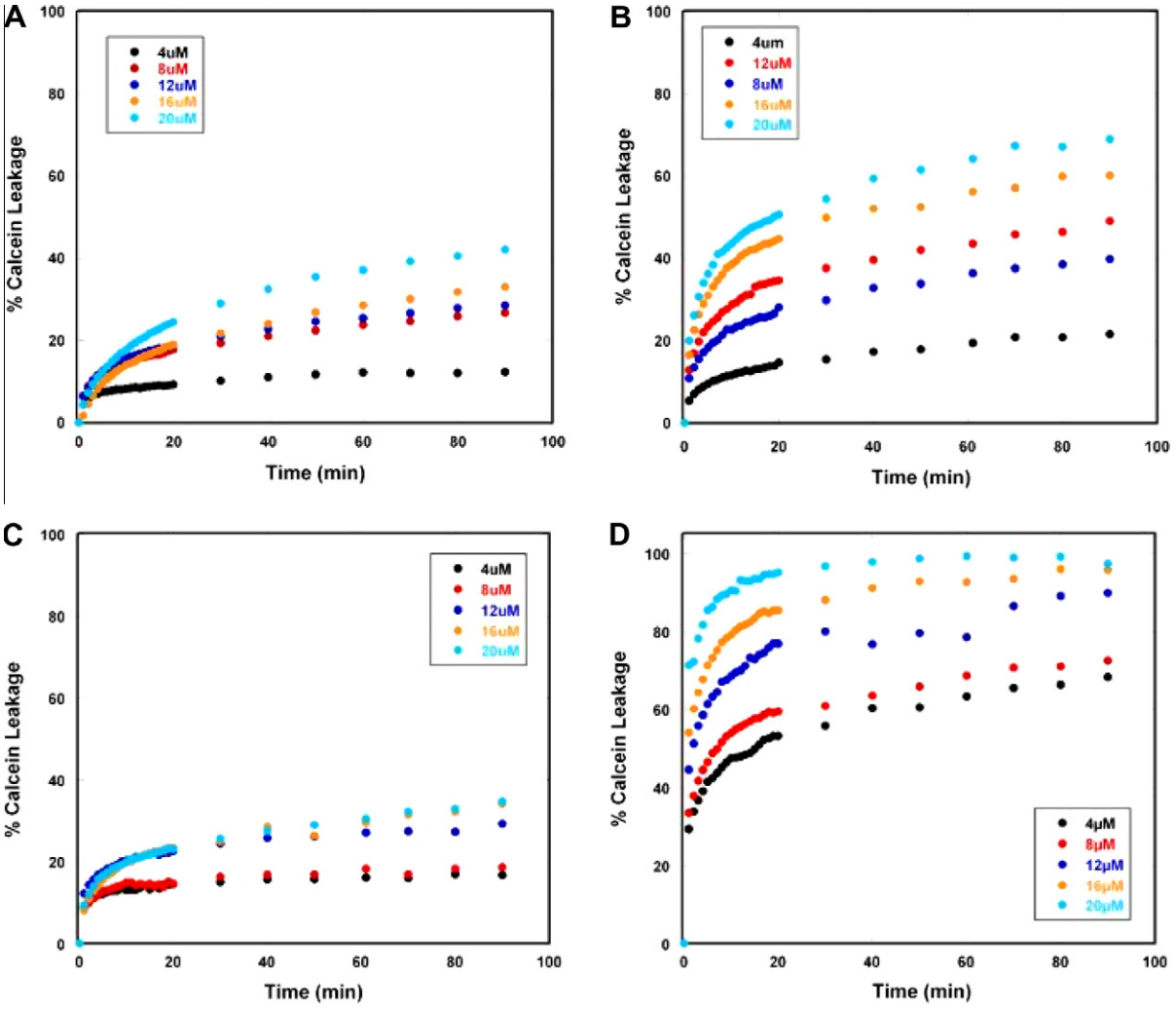

Figure 2. The time dependent release of calcein from 4:1 POPC/POPG LUVs induced by increasing concentrations of compounds **23** (A), **64** (B), **39** (C), and **61** (D) as measured by fluorescence.[30–32] The unit of time is minutes.z

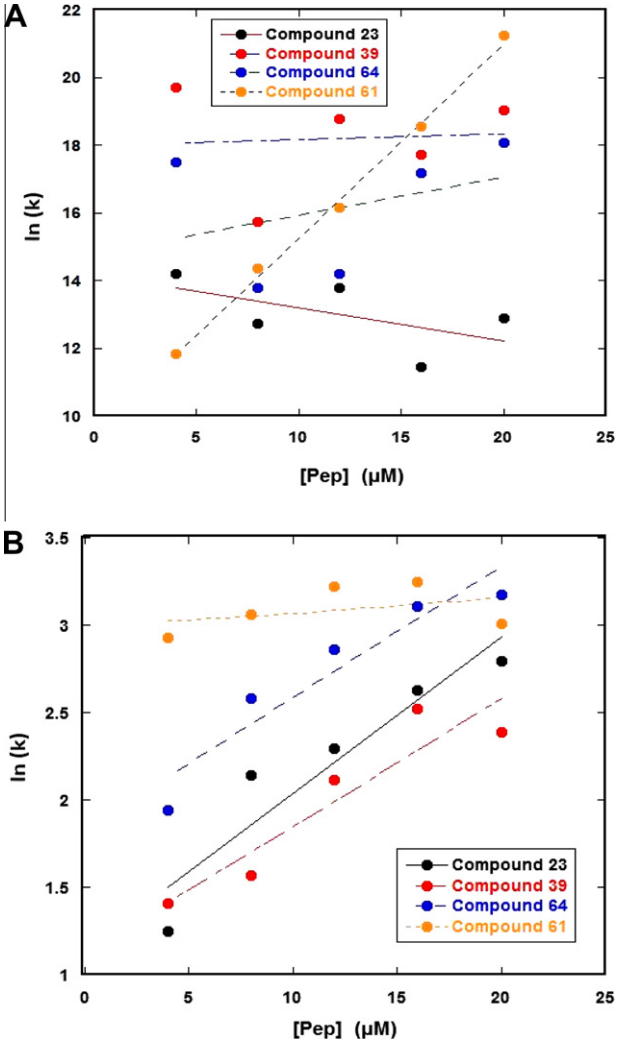

Figure 3. Plot of the ln(k) of the initial induced calcein leakage verse peptide concentration in from (A) POPC liposomes and (B) 4:1 POPC/POPG liposomes.

Table 4
Analysis of the initial rate of calcien leakage as function of concentration

Compound	Lipid:POPC		Lipid 4:1 POPC/POPG	
	k	R	k	R
23	9.7×10^{-2}	0.581	8.9×10^{-2}	0.939
39	1.1×10^{-3}	0.36	7.3×10^{-2}	0.934
61	5.7×10^{-1}	0.998	8.5×10^{-3}	0.398
64	1.4×10^{-2}	0.06	7.5×10^{-2}	0.948

1, Compounds **64** (three Lys residues at the C-terminus) and **61** (four Lys residues at the N-terminus) induced the most leakage. The decrease in the electric charge in compound **64** could allow for the peptide to maneuver around the positive charges of the head groups more easily, thus increasing the binding and causing increased leakage. A possible explanation for the increase in leakage observed for compound **61** is that the N-terminus with the four Lys residues will be associated with the polar lipid head groups on the surface of the membrane, allowing the C-terminus with the hydrophobic Tic residue to insert deeper into the bilayer, which consequently, would induce more leakage.

Peptide induced leakage of the 4:1 POPC/POPG LUVs was determined to be concentration dependent as can be seen in Figure 2.

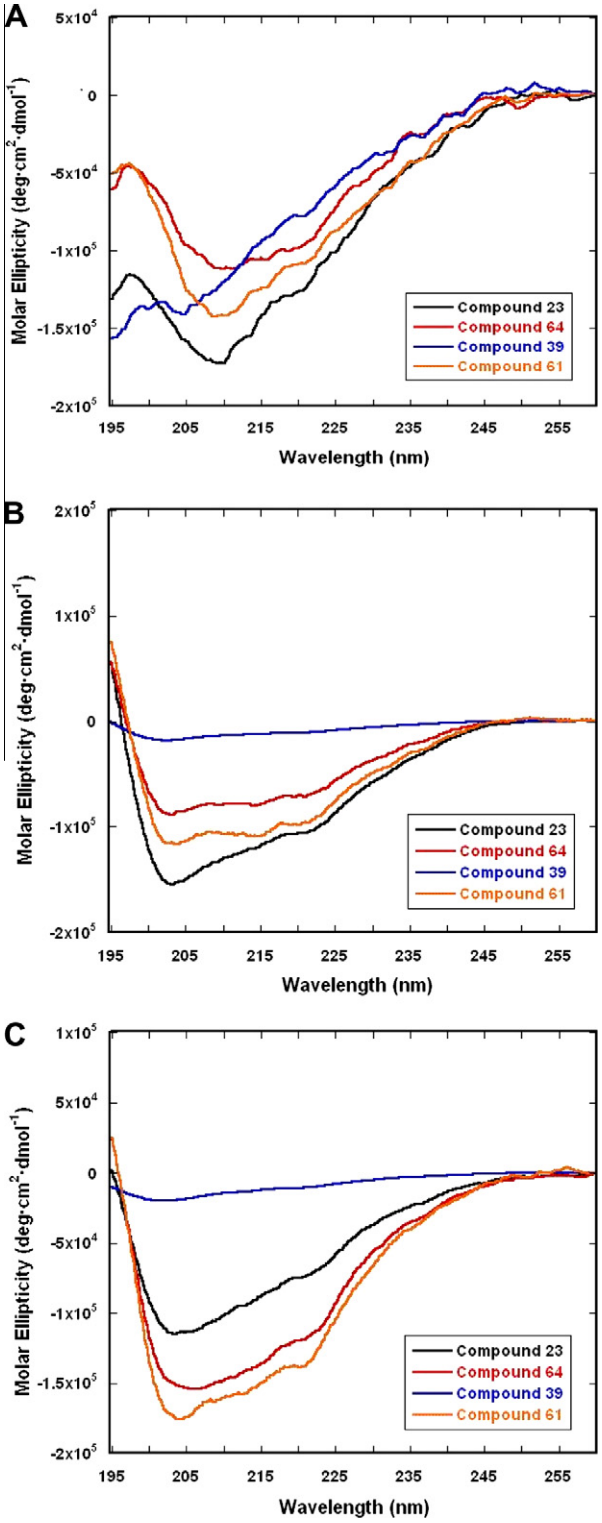

Figure 4. Far-UV CD spectra of compounds **23**, **64**, **39** and **61** (1 mg/mL) in (A) 40 mM phosphate buffer, pH 6.8. (B) 80 mM SDS and (C) 80 mM DPC in 40 mM sodium phosphate buffer, pH 6.8.

Compounds **23** and **39** did not exhibited significant increase in the leakage as the peptide concentration increased as was the case with compounds **61** and **64**. The increased leakage of compounds **64** and **61** is consistent with the increased antibacterial activity of these compounds compared to compound **23**. Clearly these peptides are members of the general class of membrane disrupting antimicrobial peptides.

Close examination of the initial rates of calcein leakage shown in Figures 1 and 2 indicate that these compound exhibit different rates of calcein leakage from POPC and 4:1 POPC/POPG liposomes. The initial rates of calcein leakage at each different peptide concentration were calculated and plotted as a function of peptide concentration are given in Figure 3A for POPC and in Figure 3B 4:1 POPC/POPG liposomes.. The apparent concentration calcien leakage constant k is given in Table 4 with the corresponding correlation coefficient R for the line fitting. It is clear from this data that compounds **23**, **39**, and **64** don't induce calcein leakage in a concentration dependent manner from POPC liposomes, while compound **61** does induce calcein leakage in a concentration dependent manner. The opposite behavior was observed for these compounds with 4:1 POPC/POPG liposomes. There are two proposed mechanisms for the induced leakage of the contents of liposomes by antimicrobial peptides, 'all or nothing' or 'graded' leakage.[65–70] Overall in our opinion this data suggests that compounds **23**, **39** and **64** induce calcein leakage from POPC liposomes predominately via an 'all or nothing' mechanism and induce calcein leakage from 4:1 POPC/POPG liposomes via a 'graded' mechanism. This data suggests that compound **61** induces calcein leakage from POPC liposomes predominately via a 'graded' mechanism and induce calcein leakage from 4:1 POPC/POPG liposomes via an 'all or nothing' mechanism. A conclusive statement about the exact mechanism of leakage can't be made without conducting fluorescence requenching experiments.[67–69]

The concentration dependence observed with 4:1 POPC/POPG LUVs suggests a transient pore forming mechanism.[50,71–73] During the initial bilayer insertion step, the transient pore undergoes a nucleation step, which is followed by transient 'restabilization'[71] of the lipid–peptide structure.[71] The net result is a leveling of the observed leakage after a period of time unique to each peptide.[71,74]

3.2. CD studies

CD spectroscopy is very sensitive and its use to monitor conformational changes in peptides and proteins is well documented.[55,75,76] Traditionally SUVs have been employed almost exclusively to investigate the binding of peptides and proteins with lipids in CD studies in order to minimize the contribution of light scattering on the spectra.[55,77]As pointed out by the independent work of Schmidt and co-workers[78] and Wong and co-workers,[79] SUVs due to high vesicle curvature are metastable and produce 'anomalous peptide partitioning'.[55,57,56] However, the work of Ladokhin and co-workers have shown that undistorted CD studies can be obtained in the presence of up to 3 mM LUVs above 200 nm and in the presence of 7 mM LUVs above 215 nm.[55] In this study we are employing both SUV's and LUV's to investigate the binding interactions that occur between these AMPs and a zwitterionic and an anionic liposome. In addition, in this investigation we will use anionic (SDS) and zwitterionic (DPC) micelles to isolate and investigate the surface binding interaction of these peptides from the aggregation and pore forming interactions that occur on binding to lipids.

In the analysis of CD data it must be noted that the spectrum of a peptide represents the linear combination of a number of different conformers.[75,80] This is particularly true when liposomes are used as there are several different peptide–liposome interactions possible depending on the peptide to lipid ratio. Changes in the intensity or shape of the CD spectrum of a peptide in the presence of a liposome indicate that the peptide is adopting different conformations on interacting with that particular liposome as compared to another environment such as a buffer.[75,80] Due to high percentage of unnatural amino acids incorporated into the peptides under investigation here, no quantitative estimation of secondary structural features are possible.

3.2.1. Micelle CD studies

Micelles are excellent models to study the surface interactions between peptides and membranes since micelles do not form bilayers and therefore the peptides can't fully insert into the hydrophobic core of the micelle as they can with phospholipids. For this investigation DPC micelles[29] were selected as a simple model for zwitterionic lipids of eukaryotic cells and SDS micelles[28] as a simple model for anionic lipids of prokaryotic cells. The logic behind this investigation is that the surface interactions are the origin of organism selectivity and potency. Therefore, understanding these interactions is the first step in the development of more selective and potent peptide based antibacterial drugs.

Transitioning from a buffer (Fig. 4A) environment to SDS micelles (Fig. 4B), the CD spectra exhibited two obvious changes. A shift from the λ_{max} at 195–190 nm accompanied by an increase in the intensity the λ_{max} was observed for compounds **23**, and **61**, however only a minimal increase in the intensity of the λ_{max} was observed for compound **39**. The order of the intensity of the λ_{max} decreases in the order of compounds **23**, **61**, **64** and **39**. There was also a shift of the λ_{min} at approximately 210–202 nm the intensity of the minima followed the same decreasing order of compounds **23**, **61** then **64**. For compounds **23**, **61** and **64** there is also an indication of a possible second minima at approximately 220 nm.

The CD spectra of these peptides in the presence of DPC micelles (Fig. 4C), again exhibited a shift in the λ_{max} from 195 to 190 nm

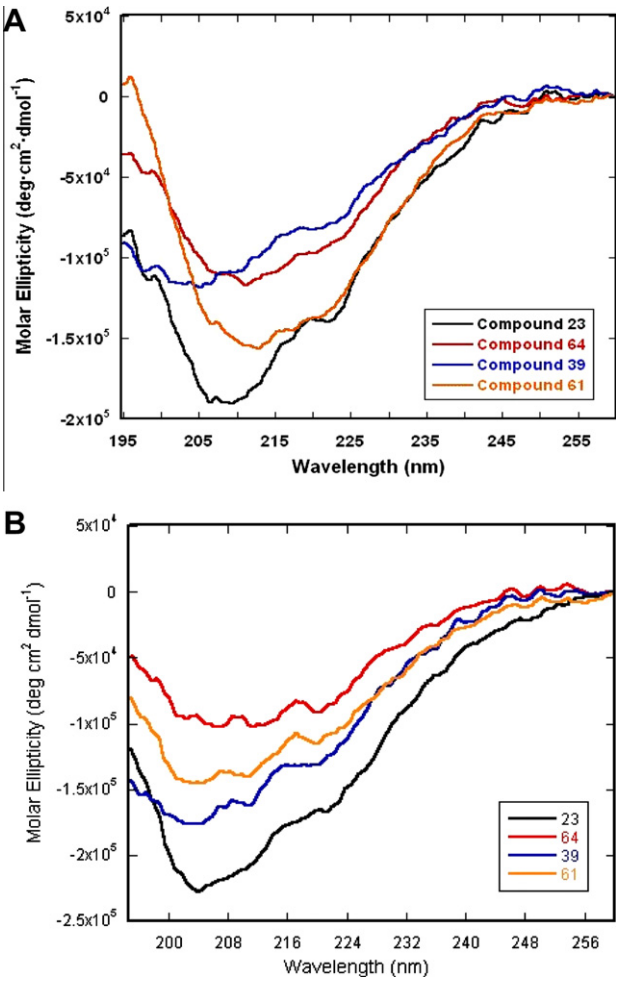

Figure 5. Far-UV CD spectra of 75 μM compound **23** and 100 μM solutions of compounds **64**, **39**, and **61** in (A) 1.75 mM POPC LUVs in 40 mM phosphate buffer, pH 6.8.and (B) in 1.75 mM POPC SUVs in 40 mM phosphate buffer, pH 6.8.

accompanied by an increase in the intensity of the λ_{max}. The order of the intensity of the λ_{max} is different from that observed in the presence of SDS micelles. There was also a shift of the λ_{min} at approximately 210–202 nm, the intensity of the minima followed the same decreasing order of compounds **61**, **64** then **23**, this is opposite of the order observed in the presence of SDS micelles. For compounds **23**, **61** and **64** there is also an indication of a possible second minima at approximately 220 nm. The observed changes in the intensities of the CD spectra of these compounds leads to the conclusion that the peptides adopt different conformations with different binding affinities on interacting with DPC and SDS micelles.[81–84] As previously stated the origin of species selectivity is based on a peptide interacting with anionic and zwitterionic membrane surfaces differently. This observation supports the hypothesis that these compounds exhibit the potential to be therapeutically useful as antibiotic drugs.

3.2.2. CD spectra of peptides in POPC LUV and SUV environments

The CD spectra of compounds **23**, **39**, **61** and **64** (100 μM concentration) in the presence of 1.75 mM POPC LUVs are given in Figure 5A. These spectra indicate that POPC LUVs induced significant changes in the shapes and intensities in the CD spectra of all peptides, including compound **39**, when compared to their corresponding spectra in buffer and in the presence of DPC micelles. One of the major differences is the complete loss of the λ_{max} at approximately 195 nm. The λ_{min} for these compounds also shift. The order of the intensity of the λ_{min} observed in presence of POPC LUV (**23** > **61** > **39** > **64**) is opposite that observed in the presence of DPC micelles. These differences indicate that the conformations adopted on binding to POPC LUVs are not only different from each other but they are also different from those conformations adopted on binding to DPC micelles indicating a more complex binding interaction.[76,83,85,86]

The CD spectra of compounds **23**, **39**, **61** and **64** (100 μM concentration) in the presence of 1.75 mM POPC SUVs are given in Figure 5B. These spectra indicate that POPC SUVs induced significant changes in the shapes and intensities in the CD spectra of all peptides, including compound **39**, when compared to their corresponding spectra in buffer and in the presence of DPC micelles.

In Figure 6 the CD spectra of each compound in the presence of POPC SUVs and LUVs are compared, and in most cases the CD spectra are different. This order of the intensity of the λ_{min} of compounds **39** and **61** are inverted in the presence of LUVs and SUVs. As pointed out by Ladokhinand co-workers[55] differences in the CD spectra of membrane associating peptides bound to SUVs and LUVs is to be expected. The comparison of their spectra are complicated by various factors including: (1) possible differences in the structures of the SUVs and the LUVs that may affect the conformations adopted by the bound peptide,[55,57] (2) possible differences in

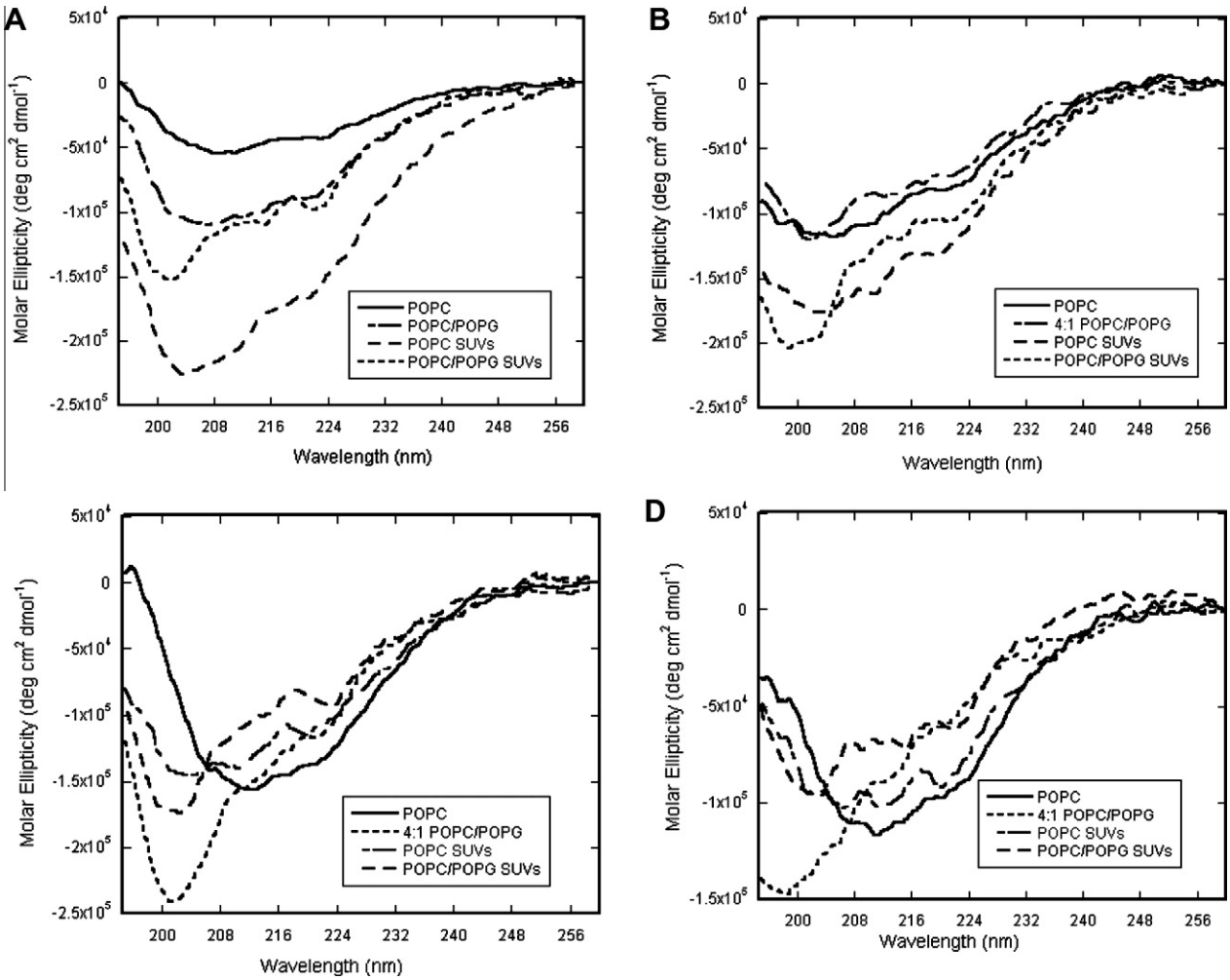

Figure 6. Far VU CD Spectra of (A) compounds **23**, (B) **39**, (C) **61** and (D) **64** in the presence of 1.75 mM POPC LUVs, POPC SUVs, 4:1 POPC/POPG SUVs and 4:1 POPC/POPG LUVs in 40 mM phosphate buffer, pH 6.8.

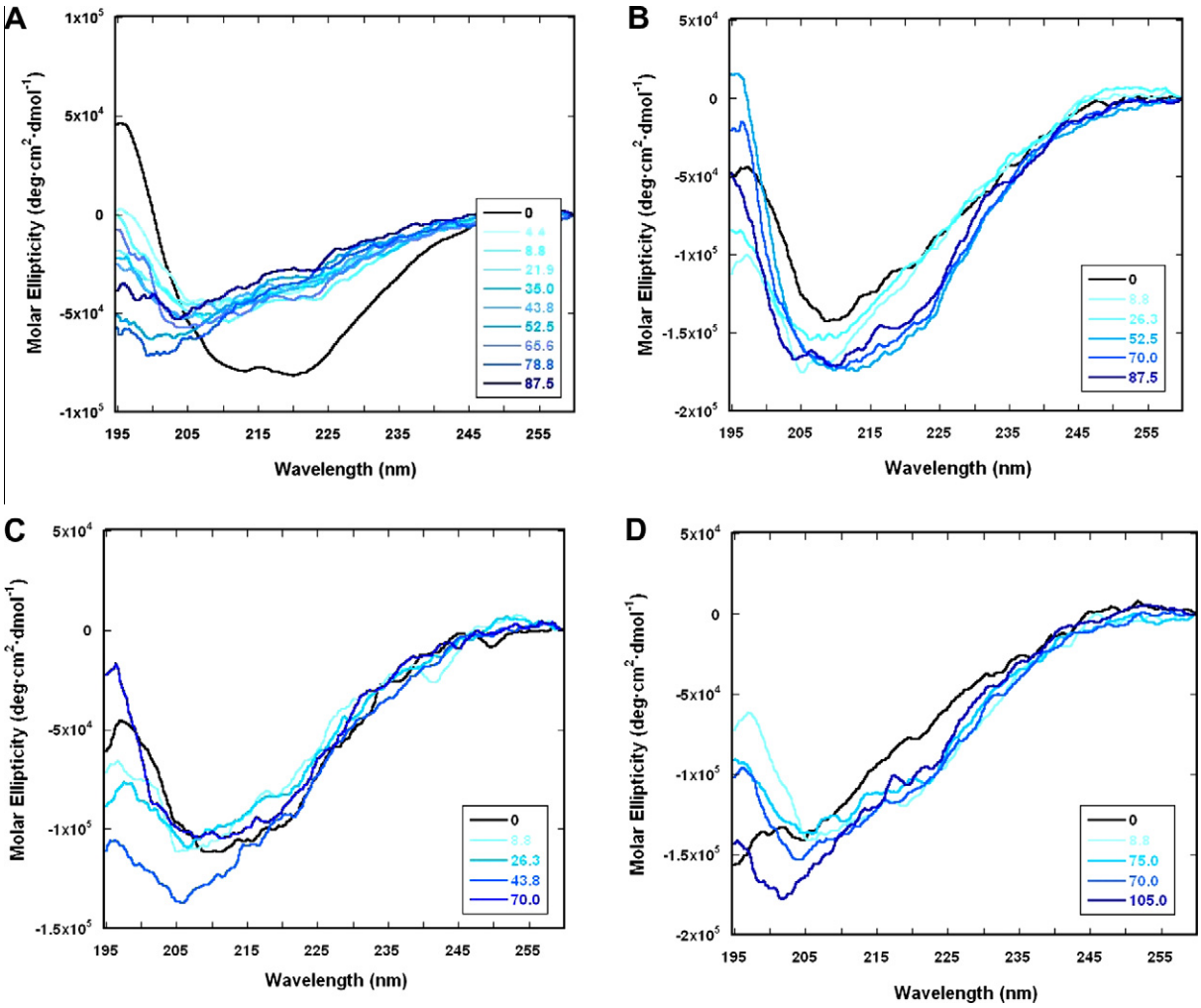

Figure 7. Far-UV CD spectra of separate samples of 200 μM solutions of compounds **23** (A) and **61** (B) and 100 μM solutions of compounds **64** (C) and **39** (D) with increasing concentrations of POPC LUVs in 40 mM phosphate buffer, pH 6.8.[16,17]

the binding affinity of the peptide for SUVs compared to LUVs, SUVs are known to exist as non-equilibrium systems that produce artificially high binding affinity,[57] (3) the light scattering of LUVs at high lipid concentrations,[57] and (4) other factors including lipid packing density and membrane curvature also play a role.[87] Huang and co-workers[87] as well as Ladokhin and co-workers[55] reported that the appearance of the CD spectra of SUV and LUV bound peptides may be different.

As seen in Figure 6, the CD spectra of compound **23** in the presence of POPC SUVs and LUVs are different in both shape and intensity. The spectrum in the presence of POPC SUVs is much more intense than the spectrum in the presence of POPC LUVs. Thus the binding conformations and the effects of the binding interactions with SUVs and LUVs are different. This indicates that the curvature or other structural features of the SUVs play a major role in determining the binding conformation and binding affinity of compound **23**. The CD spectra of compound **39** in the presence of POPC SUVs and LUVs exhibited very similar shapes but very different intensities. This data suggests that the curvature of the SUV plays a major role in determining the effects of the binding interactions of the peptide. The CD spectra of compound **61** exhibit different shapes but similar intensities, suggesting that on binding to SUVs and LUVs compound **61** adopts different conformations. The CD spectra of compound **64** adopt very similar shapes and intensities on binding to POPC SUVs and LUVs indicating that compound **64** adopts similar conformations with similar effects of the binding interactions with POPC SUVs and LUVs.

It has been well documented that the peptide to lipid ratio plays a major role in determining pore formation.[37,88,89]The work of Huang and co-workers has shown that the S-state (surface state) binding leads to a thinning of the membrane and once a 'critical peptide to lipid ratio'[37,88] is reached membrane disruption and pore formation is initiated.[37,88,89] At this critical concentration the AMPs are believed to self-assemble to form complexes of 4–6 AMPs.[63,90] These oligomeric complexes[88] are thought to insert into the hydrophobic core so that the long axis of the peptide is now oriented perpendicular to the membrane surface and the peptide is now in the I-state (inserted state).[63,90] This reorientation may be driven either entropically by the hydrophobic effect or enthalpically by Van-der-Waals Forces.[52] The 'pseudo' titration of POPC LUVs into these peptides did not yield any results that would suggest a concentration dependent interaction, as can be seen in Figure 7. The number or placement of the lysine cluster does not appear to dramatically change the spectra as the concentration of POPC LUVs increased.[75,81–84]

3.2.3. CD spectra of peptides in 4:1 POPC/POPG LUV and SUV environments

The CD spectra of these four peptides in the presence of 1.75 mM 4:1 POPC/POPG LUVs are given in Figure 8A and are very

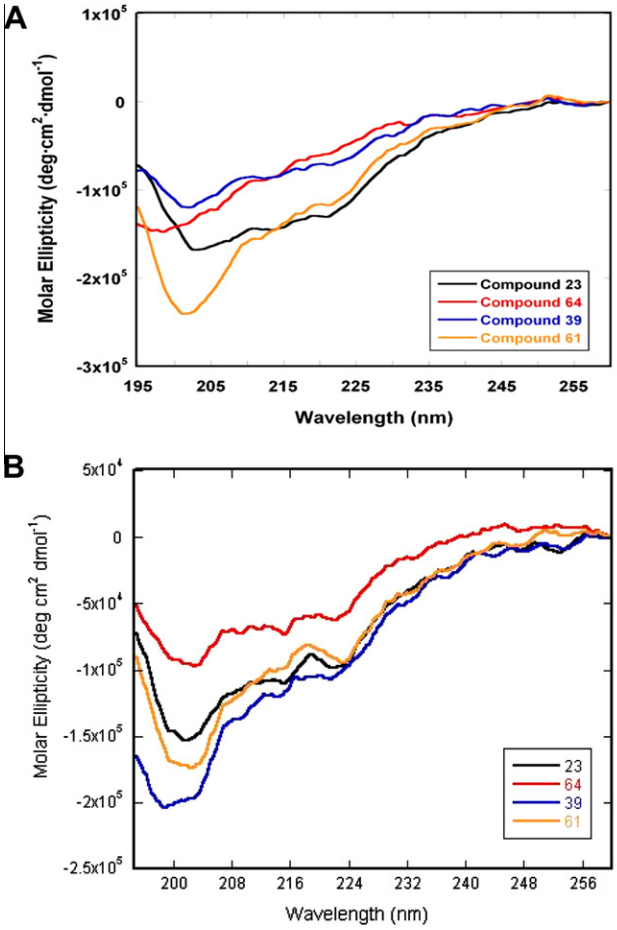

Figure 8. Far-UV CD spectra of 75 μM compound **23** and 100 μM solutions of compounds **39**, **61**, and **64** in (A) 1.75 mM 4:1 POPC/POPG LUVs in 40 mM phosphate buffer, pH 6.8 and (B) in 1.75 mM 4:1 POPC/POPG SUVs in 40 mM phosphate buffer, pH 6.8.

different from the CD spectra observed in the presence of SDS micelles, particularly in the dramatic decreases in the λ_{max} at approximately 195 nm, and the CD spectra in the presence of POPC LUVs. These differences in the CD spectra indicate that these compounds are adopting different conformations on binding to zwitterionic and anionic lipids. This suggests that these compounds will exhibit selectivity for bacterial cell over mammalian cells. The intensity of the λ_{min} of the CD spectra decrease in the order compound **61**, **23**, **64** and **39**. The CD spectra of compounds **61** and **64** are very different from the CD spectra of compound **23** in the presence of 4:1 POPC/POPG LUVs. The observed differences in the CD spectra of these compounds in the presence of 4:1 POPC/POPG suggests that these compounds can exhibit organism selectivity, which they do.

The CD spectra of these compounds are different in the presence of 4:1 POPC/POPG LUVs and SUVs. The shape and intensity of the CD spectra of compounds **23** and **39** in the presence of 4:1 POPC/POPG LUVs and SUVs (Fig. 8B) are different with the spectrum in SUVs being more intense. This suggests that the curvature and other structural features of the SUVs play a major role in defining the binding conformation(s) and the effects of the binding interactions of these compounds. The shape and intensity of the CD spectra of compounds **61** and **64** in the presence of 4:1 POPC/POPG LUVs and SUVs are different with the spectrum in LUVs being more intense. This suggests that the high curvature of the SUVs destabilized the binding conformation and the effects of the binding interactions of these compounds relative to the low curvature of the LUVs.

The 'pseudo' titrations of compounds **23** and **39** with the anionic liposomes 4:1 POPC/POPG LUVs are shown in Figure 9. At low lipid/peptide ratios, (Fig. 9—spectra in blue) the negative molar ellipticity of the peaks at 210 and 220 nm gradually decrease toward a 'critical point' which occurs at a lipid/peptide molar ratio of approximately 39:1 (Fig. 9—spectrum in green). Having reached the 'critical point,' the decreasing pattern reverses and the negative intensities of both peaks gradually increase (Fig. 9—spectra in red). Clearly, compound **23** interacts with 4:1 POPC/POPG LUVs in a concentration dependent manner. A maximum change in molar ellipticity occurred at a lipid–peptide ratio of approximately 39:1. With POPC LUVs, there was no concentration dependence observed in the molar ellipticity intensity of the minima at 210 and 220 nm. A similar decrease in the molar ellipticity for the antibacterial peptide PGLa with increasing lipid concentration to an end point of minimal intensity has been reported by Wieprechtand co-workers.[33] At this concentration they proposed that the peptide was completely bound to the liposome.[33] This observation is consistent with our hypothesis. Having reached the 'critical point', at a lipid/peptide ratio of approximately ~39:1, the intensity pattern reversed and the negative intensities of both peaks gradually increased. The 'pseudo' titrations of compound **39** resulted in similar results with a 'critical point' at a lipid/peptide ratio of ~61:1. Very different results were observed for the 'pseudo' titrations with 4:1 POPC/POPG LUVs for compounds **64** and **61**. As can be seen from the Figure 9, neither compound **64** nor **61** exhibited CD spectra transitioning from decreasing to increasing spectral intensity as was observed with compounds **23** and **39**. The intensity of the CD spectra of compounds **61** and **64** initially decreased with the first addition of anionic LUVs and then proceeded to increase (blue spectra) to a spectra of maximum intensity. At this point, the trend reversed and the intensity began to decrease (purple to red spectra). This data was evidence that compounds **64** and **61** interact with anionic membranes via different mechanisms than compounds **23** and **39**, which could support the observed increase in bioactivity of compounds **61** and **64**.

3.3. ITC studies

The binding interactions between these AMPs and 35 mM POPC LUVs was further investigated by isothermal titration calorimetry (ITC). The application of ITC to the study of peptide membrane interactions is well documented in the literature.[29,31,43] In an ideal case, this technique allows for the complete analysis of the thermodynamic parameters (free energy, enthalpy, entropy, binding constants and heat capacity changes) associated with the binding interactions of peptides to LUVs.[31,91] The interaction of peptides with lipid vesicles are driven by three forces: the hydrophobic effect, the coil–helix transition and non-classical hydrophobic effect.[26,33] Further, Wieprecht and co-workers[33] proposed that the binding of a peptide to a LUV is dependent on the global structural physicochemical properties including overall charge, hydrophobicity, and amphipathicity of the AMP.

3.3.1. POPC LUVs

At first inspection of the full titrations of POPC LUVs into compounds **23**, **64**, **39** and **61** (Fig. 10), their thermograms are very similar. All have endothermic components that require very high lipid/peptide molar ratios before all of the peptide is assumed to be bound to the LUVs. This high molar ratio is indicative of weak interactions with zwitterionic LUVs. The thermodynamic data, ΔH^0, K, ΔG and ΔS, for all four compounds are given in Table 5. All values confirmed weak interactions with POPC LUVs of similar magnitude. It is interesting to note that magnitude of the values for ΔH, K and ΔG correlated with the number of Lys residues at the

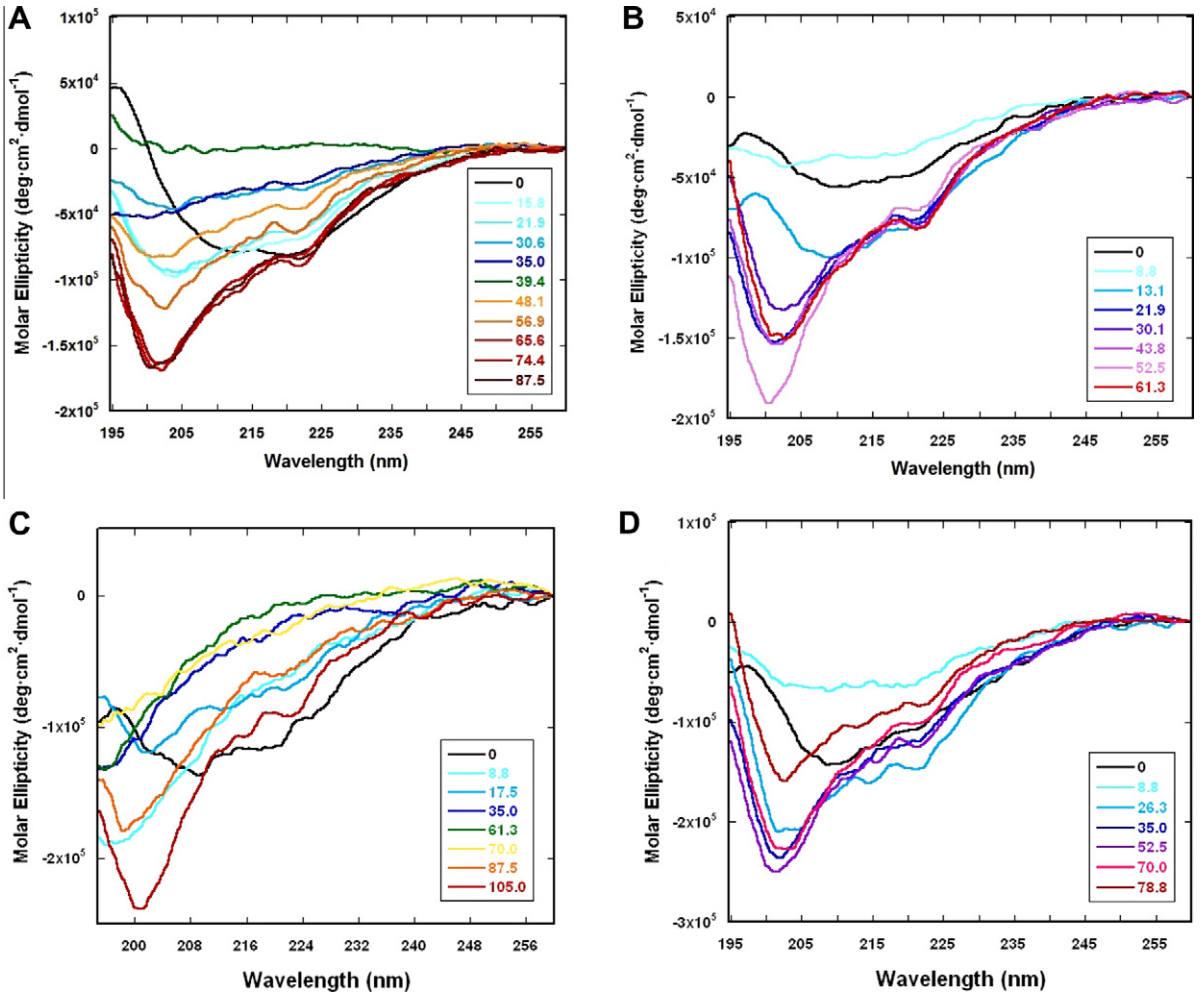

Figure 9. Far-UV CD spectra of separate samples of 200 μM solutions of compounds **23** (A), **64** (B), **39** (C) and **61** (D) with increasing concentrations of 4:1 POPC/POPG LUVs in 40 mM phosphate buffer, pH 6.8.[16,17] Color coding for compounds **23**, and **39** as the lipid to peptide ratio increases starting with a light blue color the corresponding spectra are shown in increasingly darkening blue color with decreasing spectral intensity. Until a lipid to peptide ratio is reach were the CD spectrum reaches its minimum intensity (spectrum shown in green, this spectrum represents the 'critical point'). Increasing the lipid to peptide ratio after this point causes the spectral intensity to increase and the spectra a shown in darkening shades of red. Color coding for compounds **61** and **64** are more complex since they exhibit double 'critical point' as discussed in the text.

C-terminus. As the number of Lys residues decrease the magnitude of ΔH, K and ΔG all increase.

3.3.2. POPC/POPG LUVs

The titrations of 4:1 POPC/POPG LUVs into compounds **23**, **64**, **39** and **61** (Fig. 11), result in thermograms with an initial endothermic component that transitions into an exothermic component. The thermograms obtained in the presence of 4:1 POPC/POPG LUVs are very different from the thermograms obtained in the presence of POPC LUVs indicating that these compounds interact with the two different LUVs via very different thermodynamic mechanisms. A closer examination of the thermograms for these peptides interacting with 4:1 POPC/POPG LUVs, indicate small but noticeable differences including a very small exothermic component for compound **39**, and the even smaller, almost undetectable exothermic component for compound **64**. These thermograms suggest that a slight increase and/or decrease of the charge density at the C-terminus played an important role in determining the magnitude of the exothermic component of the thermogram. This data suggest that there are at least small differences between the magnitude of the contributions of the various process that are involved in the binding of these compounds to 4:1 POPC/POPG LUVs.

Due to the complexity of the thermograms we were unable to fit thermodynamic data to an existing model. Andrushchenko and co-workers also reported similar thermograms that transition from an endothermic phase to an exothermic phase for the binding of the tryptophan-rich cathelicidin antimicrobial peptides tritrpo4 and tritrpo6 with 7:3 POPE/POPG LUVs.[35] They ascribed the observation of both endothermic and exothermic phases to various processes occurring concurrently with binding including pore formation,[33] changes in the lipid phase properties[92] or peptide aggregation.[35] They concluded that the superimposition of the binding process onto the other concurrent process makes it impossible to derive binding parameters for these types of thermograms.[35] However, by breaking down the thermograms into their separate endothermic and exothermic components it was possible to obtain insight into the different interactions that are occurring between these compounds and 4:1 POPC/POPG LUVs. The cumulative endothermic heats decreased as the number of lysines at the C-terminus increased (793, 780 and 750 cal/mol for compounds **64**, **23** and **39**, respectively).[60,93–95] An endothermic process has been attributed to pore formation[44,60] and this data suggests for these compounds pore formation is slightly more favorable with less net positive charge at the C-terminus. Formation of a pore encompasses several different processes that contribute to positive and negative ΔH values, with the positive components dominating.[60] Positive ΔH contributions include, but are not limited to, the desolvation of structured water molecules on the surfaces of

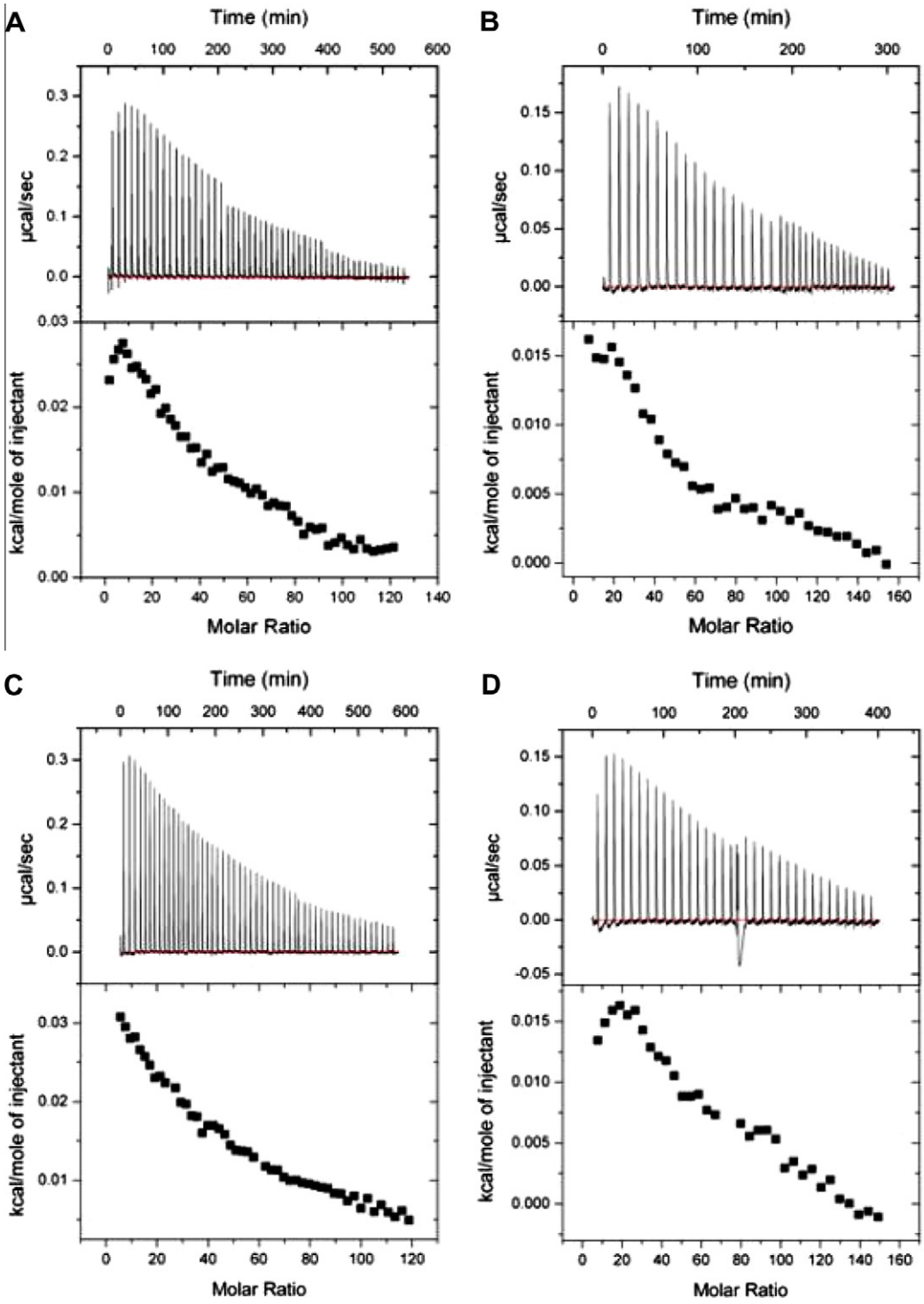

Figure 10. ITC data for the full titration of 35 mM POPC LUVs into 200 μM compound **23** (A), and 100 μM solutions of compounds **64** (B), **39** (C) and **61** (D).

the peptides and membrane, disruption of the phospholipid head groups and lipid–lipid interactions, as well as the release of the water molecules from inside the bilayer model.[26,36,41,45,95–97] Attractive electrostatic interactions, conformational changes, and reorganization of the head groups and lipid acyl chains are contributors to the negative ΔH associated with the binding process.[45,60,95] The resulting peaks observed in the thermogram after each injection reflect the dominant process, pore formation or binding, and therefore the more favorable of the two.[60]

Repositioning the cluster of Lys residues to the N-terminus as in compound **61** (Fig. 11—bottom right) produces a thermogram very similar to the thermogram of compound **23**. Both exhibited clear endothermic and exothermic components. Their cumulative endothermic ΔHs[93–95,60] were however, different with 780 and 530 cal/mol for compounds **23** and **61**, respectively. Compound **23** may have produced more 'pore forming' heat, but compound **61** bound all of the free peptide at a lipid to peptide ratio of approximately 20 compared to 36 for compound **23**.[45,94,95,60]

Table 5
Thermodynamic parameters of binding of peptides to POPC vesicles at 25 °C

Compound	ΔH^a (kcal/mol)	K^b (M^{-1})	ΔG^c (kcal/mol)	ΔS^d (cal/mol K)
23	0.67	211.0	−5.54	20.84
64	0.58	390.5	−5.91	21.78
39	0.98	168.3	−5.41	21.44
61	0.45	365.0	−5.87	21.20

[a] ΔH values are directly measured binding enthalpies and calculated using (Eq. 2).
[b] Binding constants were generated from the lipid-into-peptide titration using as described in the text.
[c] Free energies were calculated using (Eq. 3).
[d] Entropy was calculated using (Eq. 4).

The sum of their exothermic components, however, was very similar in magnitude at approximately −371 and −378 cal/mol, respectively, for compounds **23** and **61**. The similarity of these values suggests that their abilities to bind to the membrane surface were comparable.

3.3.3. The effect of varying the ratio of anionic to zwitterionic lipid on the CD spectrum of compound 23

As previously illustrated the percentage of POPG incorporated into the cell membranes of different strains of bacterial will vary.[62] This variation in lipid content has been offered as a possible explanation of the observed differences in organism selectivity

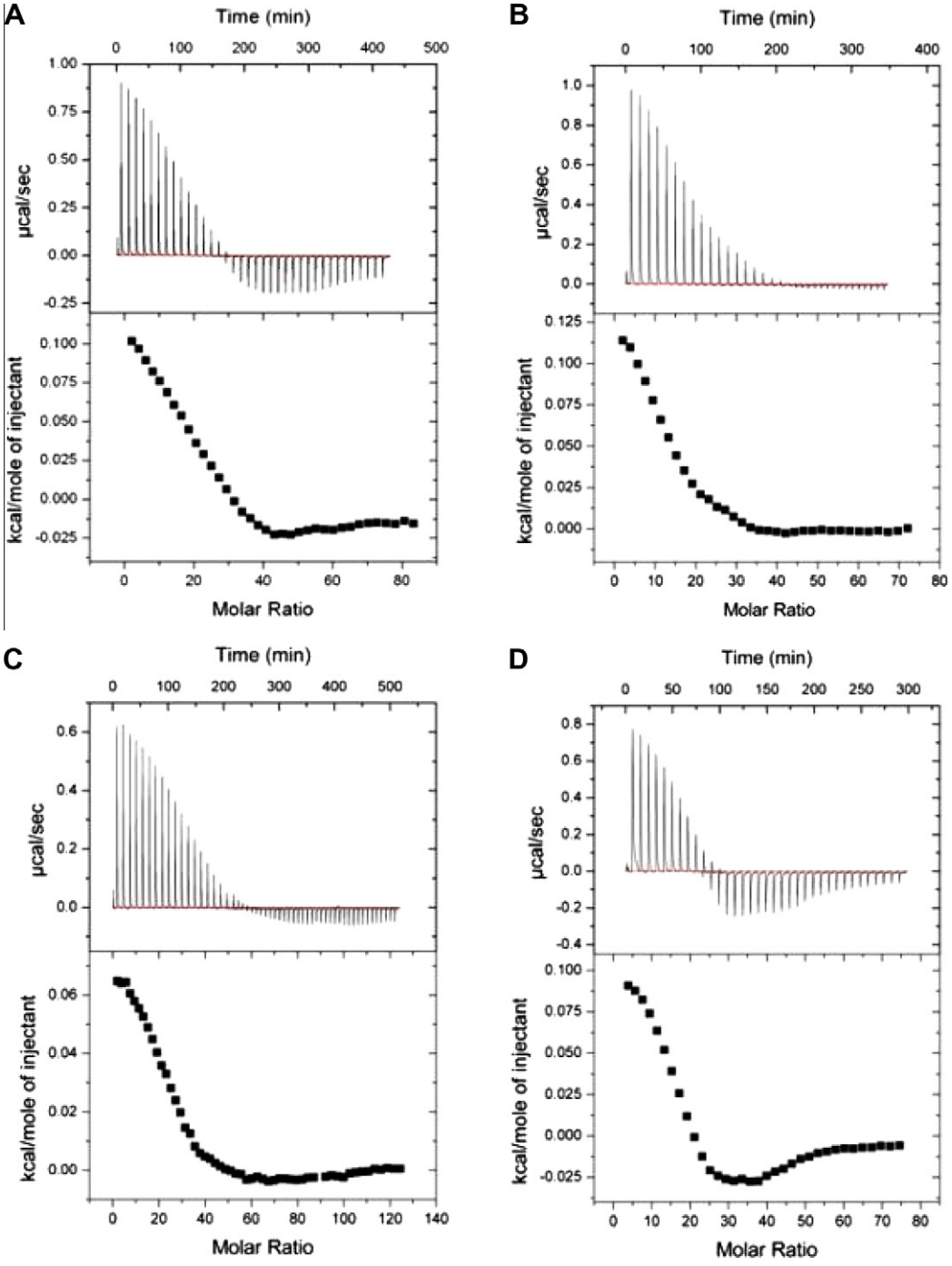

Figure 11. ITC data for the full titration of 35 mM 4:1 POPC/POPG LUVs into 200 µM solutions of compounds **23** (A), **64** (B), **39** (C) and **61** (D).

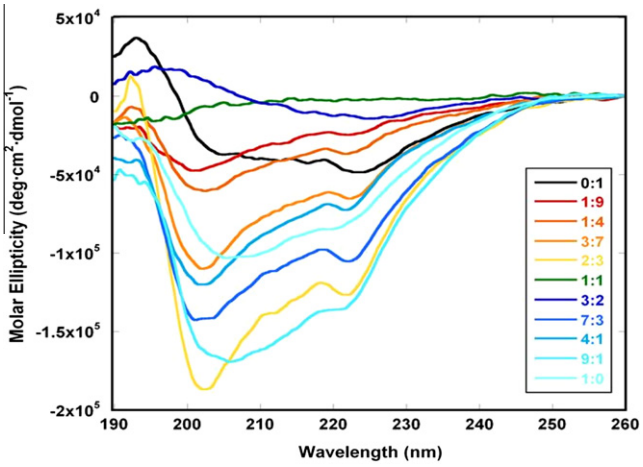

Figure 12. Far-UV CD spectra of compound **23** in presence of different ratios of zwitterionic POPC and anionic POPG lipids. Spectra increase by increments of 10% from a ratio of 0% POPC/100% POPG (black spectrum) to a ratio of 100% POPC/0% POPG (light blue spectrum).

and potency for these compounds. To evaluate whether this hypothesis could be correct, the following CD data was collected. As seen in Figure 12 the CD spectra of compound **23** as you go from a ratio of 0% POPC/100% POPG (black spectrum) to a ratio of 100% POPC/0% POPG (light blue spectrum) change dramatically. These changes indicated that as the percentage of anionic lipids changes so does the lipid-bound conformation of compound **23**. This is consistent with our hypothesis explaining the observed differences in the antibacterial activity of these compounds.

4. Conclusions

It is very difficult to correlate the observed spectroscopic and thermodynamic data in the presence of membrane model systems of these AMPs to their biological activity. What is clear is that the placement and total charge density plays a major role in defining how these AMPs interact with membranes and their resulting biological activity. This study has demonstrated: (1) These AMPs interact with anionic and zwitterionic membranes via different mechanisms. The best approximation to a known mechanism of action suggests that these peptides interact with POPC LUVs via some type of surface interaction similar to the 'carpet' mechanism.[71] While the best approximation to a known mechanism of action suggests that these peptides interact with 4:1 POPC/POPG LUVs via some type of pore formation.[50,71,72] (2) There are detectable differences between how each of these AMPs interact with the membrane models, that is position and total charge density defines how these AMPs interact with specific membrane models and thus the resulting biological activity. (3) The curvature and other properties of SUVs effect the conformations and binding affinity of these AMPs.

Acknowledgments

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