Rational Design of Vector and Antibiotic Peptides Using Solid-State NMR

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Abstract: The application of ^{2}H solid-state NMR in determining structure activity relationships and mechanism of action of membrane active peptides is discussed. The enhancement of the disruption of anionic lipids in the membrane by new lead compounds is shown to be a key determinant of both DNA vector and antimicrobial activity.

Key Words: Solid-state NMR, gene transfer, antibiotic peptides, histidine, LAH4, pleurocidin.

INTRODUCTION

Cationic amphipathic helical peptides have been shown to have important DNA vector and antimicrobial activities and have the potential to be important tools in both pharmaceutical and biotechnological arenas. The fulfilment of this potential very much depends on the ability of researchers to improve the activity of either naturally occurring peptides or model compounds, so that lead compounds can become competitive and cost effective additions to the respective arsenals. Since the interactions between these amphipathic peptides and biological membranes plays a crucial role in determining their activity, suitable methods are required to analyse these interactions which determine the most important structure-activity relationships that must be considered in designing new lead compounds. The ideal technique would be relatively quick in nature to allow screening of multiple drug candidates. It should be able to distinguish between selected components in the membrane with differing properties and it should be sufficiently sensitive to allow differential effects of similar peptides to be distinguished. Since the peptides are rather small in nature, any probe that is added to the system should be non-perturbing to the activity of the peptide whilst, finally, the technique should contribute to the understanding of the peptide activity by being readily comparable with other techniques and the *in vitro* characteristics.

Solid-state NMR is emerging as a powerful technique to study biological aggregates and provide structural information intractable by more traditional techniques. In particular it has been very successful in determining the structure of biological fibrils such as those implicated in degenerative diseases such as Alzheimer's or Parkinson's diseases [1, 2] and is now being directed at determining the structure of cations [3], small molecules [4, 5], and peptide hormones [6, 7] bound to membrane proteins. Solid-state NMR has a considerable advantage in that it can be applied to both the protein and lipid components of a biological membrane. In this respect, the observation of signals from either natural abundance or labelled lipids has contributed greatly to our understanding of how certain classes of peptide interact with biological membranes. Having introduced the various NMR tools available for studying the lipid component of biological membranes, we show how and why we have selected one particular method, ^{2}H NMR of chain perdeuterated lipids, to investigate the interaction of histidine rich amphipathic helical peptides with a variety of model membranes [8, 9]. Using this method, we have shown that one of the crucial determinants of the peptide activity involves the interaction between the peptide and the anionic lipid component of the membrane [8]. We then show how we were able to screen new candidate peptides for promising membrane destabilising capabilities resulting in new lead compounds with enhanced activities [8, 10]. We conclude that application of solid-state NMR to the rational design of DNA carriers [8, 10] and antimicrobial peptides [9] delivers a high level of detailed information and our particular method satisfies the conditions set down above constituting an effective screen for future biological activities.

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NMR TOOLBOX

When considering the study of a peptide in a membrane environment using solid-state NMR, two differing approaches can be taken; firstly one could devise methods that probe the structure, conformation and dynamics of the peptide in a range of environments. This approach is widely used when a detailed study of a single peptide of interest is envisaged but when a comparison of many candidate peptides is desired such an approach is hindered by a number of factors. Labelling considerations such as the cost of labelling multiple peptides with stable isotopes, the design of the labelling scheme when resolution of multiple labelled sites can be problematic especially in a designed peptide with little variation in sequence, and the relatively poor sensitivity of solid-state NMR methods all contribute to reduce the suitability of this approach for our purposes. Alternatively we might consider the effect of a peptide on its lipid environment. Using this approach we could devise a labelled membrane system which remains constant for each experiment, is relatively easy to prepare and enhances sensitivity due to the larger number of molecules being studied and the generally sharper linewidths of the signals from the more mobile lipids.

Having decided to study the lipid component of a model membrane there are a number of differing solid-state NMR techniques that are available and can be selected on the basis of the information that is required [11]. Solid-state NMR of membrane lipids can essentially provide three kinds of information (summarised in Table **1**); firstly, anisotropic interactions, such as the chemical shift anisotropy (CSA), are a useful indicator of the morphological state of a membrane. For example, natural abundance ^{31}P NMR of the phospholipid headgroup has been used to give a clear indication of whether a membrane remains intact upon addition of a given peptide [12, 8]. The broad powder pattern observed for randomly oriented bilayers gives way to sharp upfield resonances upon conversion to oriented bicelles or wormlike sheets whilst a sharp resonance at the isotropic chemical shift value is expected for lipids in a micelle [12]. Secondly the electrostatic interactions involving the lipid headgroups can be probed using NMR as a "molecular voltmeter". This has been performed in a number of ways, either using ^{2}H headgroup labelled lipids where the observed quadrupolar splitting is dependent on the relative orientation of the P-N dipole in the bilayer and hence the charge or charge distribution [13], or naturally abundant isotopes where the surface charge is reflected in the isotropic chemical shift (^{31}P, ^{14}N) under magic angle spinning (MAS) conditions [8, 15, 15b] or the size of the quadrupolar coupling (^{14}N) [15]. Finally, the location of the peptide, or other drug, in the membrane can be determined either directly, by measuring peptide-lipid contacts using MAS NOESY or spin diffusion experiments [16] or indirectly by considering the effect of the peptide on the hydrophobic interior of the membrane, constituted by the

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Table 1. A Comparison of NMR Active Nuclei Commonly Present in Lipid Membranes and the Information that is Tractable by Solid-State NMR Methods

Nucleus	Labelling	Interaction	Technique/Information (Time Domain)
^{1}H	Natural abundance	Nuclear Overhauser Effect	MAS/NOESY gives direct measurement of peptide lipids contacts
^{2}H	Headgroup	Quadrupolar coupling (10-100 kHz)	Molecular voltmeter
	Acyl chain		Order parameter profile (μs)
		Relaxation (T_1, T_2)	Acyl chain dynamics (T_1: ns→μs; T_2: ms)
^{13}C	Natural abundance	^{1}H-^{13}C dipolar coupling (2-20 kHz)	Order parameter profile (μs)
^{14}N	Natural abundance	Quadrupolar coupling	Molecular voltmeter
^{31}P	Natural abundance	Chemical shift anisotropy (5-50 ppm)	Membrane morphology (ms→μs)
		Isotropic chemical shift	Molecular voltmeter
		Relaxation (T_1, T_2)	Headgroup dynamics (T_1: ns→μs; T_2: ms)

lipid acyl chains. This can be observed either in terms of the effect of the peptide binding on NMR relaxation parameters [17] or by quantifying the alteration of order parameters. These order parameters can be provided by measuring either the size of ^{1}H-^{13}C dipolar couplings for different segments in the acyl chain [18] or ^{2}H quadrupolar couplings in isotope enriched lipids [19] which are available commercially [20]. We have used a number of these methods in our laboratory but will focus here on the application of ^{2}H NMR of chain perdeuterated lipids to the study of membrane active cationic peptides, in particular their interaction with anionic lipids.

^{2}H NMR OF PERDEUTERATED LIPIDS

^{2}H NMR of perdeuterated lipids is a technique commonly used as a structural probe in liquid crystalline bilayers [21] to quantify the perturbation of phospholipid bilayers by peptides. In particular, averaging of the deuterium quadrupolar interaction is sensitive to motions in the order of ~10-100 kHz [11]. For chain deuterated lipids incorporated into unoriented liposomes, the rigid limit quadrupolar coupling for a deuteron in a C-D bond (168 kHz) [19, 22] is averaged to a value of between 33 and 26 kHz for the CD_2 group closest to the lipid headgroup, depending on the lipid composition in the liposome [23]. For CD_2 groups further down the lipid fatty acyl chain the quadrupolar splittings are averaged further such that when the quadrupolar splittings corresponding to each acyl chain segment are resolved in the observed spectrum (Fig. **1A**), an order parameter (S_{CD}) profile of the lipid chain can be constructed (Fig. **1B**). For unoriented liposomes a dePakeing analysis [24] is applied to the spectrum to determine the quadrupolar splitting expected for

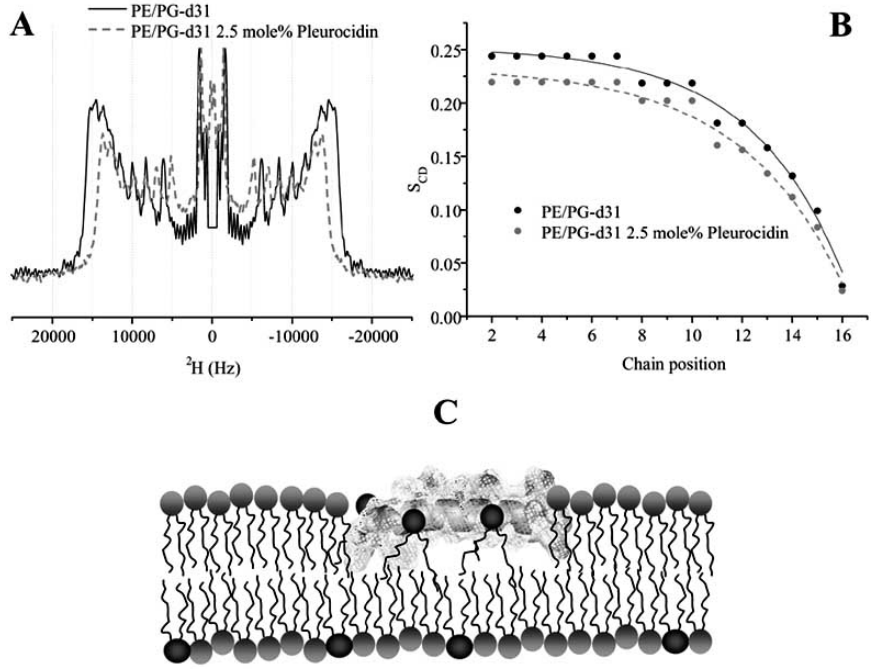

Fig. (1). ^{2}H solid-state NMR spectrum of chain deuterated POPG (d31) in POPE:POPG (3:1) vesicles showing the characteristic Pake pattern composed of quadrupolar splittings for acyl chain segments containing CD_2 or CD_3 (terminal) labelled groups (**A**). Spectra for peptide free vesicles (Solid line) and vesicles in the presence of 2.5 mole% pleurocidin are shown. The resulting order parameter profile (**B**) reveals the disruption of the lipid acyl chains by the peptide which is *proportionally* greater in the lower half of the chain. A model for the interaction of the peptide shows the peptide preferentially interacting with, and disrupting anionic lipids in the mixed membrane (**C**).

a macroscopically oriented sample. The order parameter profile is then used as a basis for assessing the effect of binding of the peptide, drug or other lipid of interest. In many cases a reduction in the size of the quadrupolar couplings and hence order parameters is observed but specific binding of a peptide to one lipid component [25, 26] or the presence of a sterol [26, 27] can cause an ordering of the labelled lipid. Here we show how we have used this technique to identify the interaction of cationic peptides with the anionic lipid component as a crucial determinant of activity. We will describe a number of peptides that are all rich in histidine residues and are oriented parallel to the membrane surface in their active conformation [28-30]. Initially we explain the principle and rationale behind our approach using the example of pleurocidin, an antimicrobial peptide derived from the Winter Flounder, *Pleuronectes americanus* [31] and then we will show how we have used the technique in understanding structure activity relationships in designed histidine rich peptides with antibiotic and gene transfer capabilities.

PLEUROCIDIN

Pleurocidin is one of a number of cationic antimicrobial peptides derived from marine organisms [32] and in particular, flatfish [33]. Pleurocidin is a 25 residue cationic peptide that has been determined by solution NMR in dodecylphosphocholine (DPC) micelles, a membrane mimicking environment, to adopt an amphipathic α-helical structure [34]. Pleurocidin is capable of causing dye leakage from liposomes [35], translocating across model membranes [35] and demonstrates pore forming activity in planar lipid bilayers [36]. Importantly, all of these properties are greatly enhanced when the membranes are partly composed of anionic lipids. Interestingly however, sub-lethal concentrations of pleurocidin have been shown to inhibit macromolecular synthesis [37] and hence, although pleurocidin is known to depolarize membranes, it is thought to be one of a growing number of antimicrobial peptides that are proposed to have an intracellular target either in addition to, or instead of, pore formation as the major killing strategy [38]. Pleurocidin, like other flat-fish derived peptides [39, 40] is rich in histidine residues (Table **2**) and since the localization of pleurocidin expression reveals the peptide to be distributed in both mucin skin granules and intestinal goblet cells [41], it may function over a range of pH values *in vivo*. Therefore we proposed that by studying the membrane interaction of pleurocidin at both neutral and acidic pH we could determine the role of the three histidine residues in the peptide killing strategy by determining their contribution to the membrane activity of the peptide. Since the bacterial membrane is likely to remain intact at sub-lethal concentrations, we were careful to select a peptide concentration (2.5 mole%) that should not have a bilayer disruptive effect [12b] but nevertheless does cause measurable (30%) dye leakage from anionic vesicles [35] indicating that at a peptide-to-lipid ratio of 1:40 the membranes remain intact but become permeable to dye, and potentially other molecules. Having demonstrated that pleurocidin is aligned along the surface of the membrane using static ^{15}N NMR of macroscopically aligned samples [28], we prepared mixed lipid vesicles containing phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) as the zwitterionic and anionic phospholipids respectively. In each sample, a proportion (25%) of the lipids were deuterated along the length of one acyl chain and samples containing either labelled PE or PG were prepared. The ^{2}H NMR spectra obtained (Fig. **1A**) and the subsequently prepared order parameter profiles (Fig. **1B**) showed that addition of peptide during formation of the lipid vesicles caused a reduction of order in the lipid acyl chains of labelled lipids which was much more marked for the anionic PG lipids [28]. The disruption of the acyl chains was proportionally also much more evident for the lower half of the lipid, i.e. in the centre of the hydrophobic core of the membrane. Spectra obtained of vesicles prepared at acidic pH were practically identical to those obtained at neutral pH, in contrast to the effect of LAH4 discussed below, indicating that protonation of the histidine residues did not affect the membrane interaction of pleurocidin. The story that the spectra tell, therefore, is that the peptide inserts into the headgroup region, where its long axis is parallel to the membrane surface, and binds preferentially to anionic lipids inducing a increased separation of the lipids and hence a disruption of the chain order (Fig. **1C**). In a recent study a similar effect was seen for a derivative of magainin 2 (MSI-78) revealing that a reduction in the membrane thickness, as measured by atomic force microscopy, accompanies the disruption of lipid chain order [42]. Whilst the mechanism of action of pleurocidin is not clear, we have shown that the disruption of the lipid acyl chain and the likely accompanying thinning of the membrane are linked to, and are sufficient for, significant release of dye from anionic liposomes [35], implying that under these conditions the bacterial membrane should become similarly permeable. Importantly, this effect needs to be quantifiably related to the antimicrobial activity of the peptide. Pleurocidin exhibits inhibitory activities against bacteria in the micromolar concentration range being very active against *Escherichia coli*, *Bacillus subtilis*, and a range of fish pathogens and has thus been considered as an agent suitable for application in medicine and aquaculture [31]. Pleurocidin, however, is much less effective at combating other common pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* [31] and we were interested to see whether a peptide with enhanced membrane disruptive capabilities would prove to be a more robust antimicrobial agent against these common pathogens.

LAH4 AS AN ANTIBIOTIC PEPTIDE

LAH4 is a designed histidine rich amphipathic α-helical peptide [29] that has been shown to possess both antibiotic [30, 9] and DNA delivery [43, 8] capabilities. Although this peptide is rich in histidine and lysine residues like pleurocidin above, the structural design is rather different. In LAH4, the lysine residues are seques-

Table 2. A Comparison of the Peptide Sequences and Their Selected Properties for Peptides Discussed in the Text. Residues that are Positively Charged at Either Acidic or Neutral pH are Marked in Bold. All Peptides are C-Terminal Amidated Contributing +1 Charge to the Peptide

Peptide	Sequence	Peptide Length (Residues)	Nominal Charge at pH 7	Nominal Charge at pH 5
Pleurocidin	GWGSFF**KK**AA**H**VG**KH**VG**K**AALT**H**YL	25	+5	+8
LAH4	**KK**ALLALAL**HH**LA**H**LAL**H**LALAL**KK**A	26	+5	+9
LAH4-L0	**KK**ALLA**H**ALA**H**LALLAL**H**LAL**H**LK**K**A	26	+5	+9
LAH4-L1	**KK**ALLA**H**AL**H**LLALLAL**H**LA**H**AL**KK**A	26	+5	+9
LAH4-L2	**KK**ALLALAL**HH**LALLAL**H**LA**H**AL**KK**A	26	+5	+9
LAH4-AL6	**KK**ALL**H**LALALLAL**H**A**H**ALAL**H**LK**K**A	26	+5	+9

tered at the termini, whilst the histidine residues are located in the centre of the peptide and are clustered on one face such that an amphipathic helix is formed in the presence of membranes [30] (Fig **3A**). At acidic pH when the histidines are protonated the helix adopts an in-plane orientation at the membrane surface, as does pleurocidin, however at neutral pH the uncharged histidine residues can be accommodated in the hydrophobic core of the membrane and the peptide adopts a trans-membrane orientation with the lysine residues acting as anchors at the membrane surfaces [29] (Fig. **2**). Recently, we investigated the antimicrobial activity of this designed

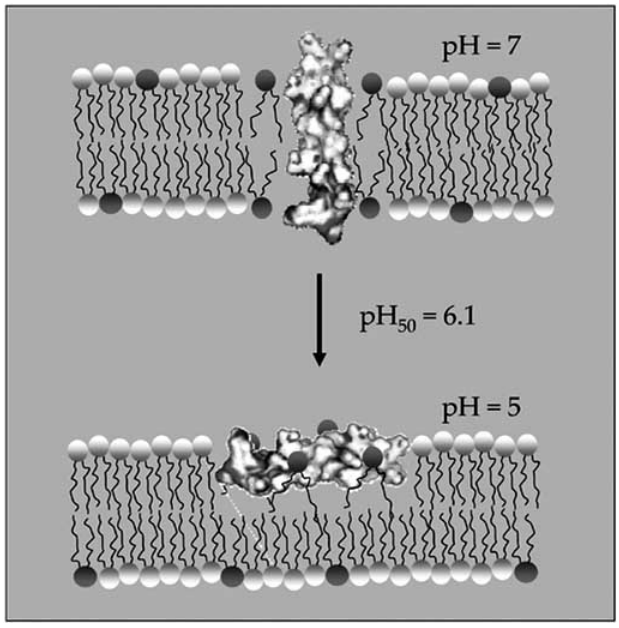

Fig. (2). A model for the interaction of LAH4-L1 with mixed model membranes, containing anionic (dark grey) and zwitterionic (light grey) lipids at neutral and acidic pH. At neutral pH the peptide adopts a transmembrane orientation and the disruption of chain order in either anionic or zwitterionic lipids is slight. Below a pH of 6.1, the peptide adopts an in-plane orientation where it becomes more active and disrupts anionic lipid acyl chains and is a much more effective antimicrobial agent.

peptide in more detail [9]. In particular, we were interested in the expected differences in antimicrobial activity at acidic and neutral pH since not only would a reduction in pH increase the nominal charge of the peptide from +5 to +9 but the orientation of the peptide in the target membrane would also be altered dramatically. Perhaps surprisingly, we found that the LAH4 peptides were effective antimicrobial agents against certain bacteria at neutral pH without apparently disturbing the membrane [9]. Whilst the origin of this activity is yet to be determined, our results are consistent with a possible intracellular killing mechanism at neutral pH as postulated for pleurocidin amongst other antimicrobial peptides [37, 38]. Interestingly, the LAH4 peptides and the more active LAH6 were only poorly effective against *S. aureus* and *P. aeruginosa* at neutral pH. However, when the pH of the medium was dropped the LAH peptides all became effective against *P. aeruginosa* and became more effective against the test bacteria *E. coli* and *Bacillus megaterium*. Importantly, the more active conformation of LAH4 and all its derivatives, like pleurocidin, is the in-plane orientation adopted at acidic pH. We therefore tested the interaction of LAH4-L1, a peptide which had antimicrobial activity representative of the LAH4 isomers but in addition was the most effective vector peptide to date [8], with anionic model membranes using the ^{2}H solid-state NMR technique described above. The results were similar to those obtained with pleurocidin with very little effect on the lipid acyl chain order of either zwitterionic PE or anionic PG being observed at neutral pH but at acidic pH, when the peptide was in the more active conformation, there was a noticeable reduction in chain order for both labelled PE and PG lipids but which was much more dramatic for the PG lipids. Noticeably, the disruption of chain deuterated PG by 2.5 mole% LAH4-L1 at acidic pH was much more effective than that of 2.5 mole% pleurocidin at either acidic or neutral pH (Fig. **3**), the likely difference coming from the nature of the amino acids mediating the membrane interaction as in LAH4-L1, the histidine residues appear to be able to interact directly with the anionic lipid headgroups whereas in pleurocidin the histidine residues appear screened from the membrane surface by the presence of lysine residues throughout the length of the peptide (Fig. **3**). It is therefore interesting to note the enhanced membrane disruptive effect in model membranes and the much greater antibiotic activity against *P. aeruginosa* in particular, of LAH4-L1 at acidic pH (<10 μM) [9] when compared with that of pleurocidin (>35 μM) [31]. On the basis of these results, it seems clear that design of histidine rich peptides, where the histidine residues directly mediate the membrane interaction even at neutral pH, can lead to the development of highly effective antimicrobial agents and such development continues in our laboratory.

LAH4 AS A VECTOR FOR NUCLEIC ACIDS

In addition to being an effective antimicrobial peptide, LAH4 also fulfils the requirements for being a peptide DNA carrier in that it is relatively short, presents cationic residues which allow electrostatic interactions with DNA but with a limited charge density, is soluble in aqueous solutions and is able to interact with and destabilise membranes. The potential for using LAH4 as a DNA vector peptide has been demonstrated against a number of cell lines with efficiencies that are comparable to other non-viral transfection agents such as polyethylenimines (PEIs) and the monocationic lipid DOTAP [43]. Whilst the transfection efficiency of the lead peptide, LAH4, from the original study is high [43], we nonetheless were keen to establish whether the efficiency and potentially also the selectivity of peptide vectors based on LAH4 could be enhanced by a rational design process. LAH4 is thought to complex with plasmid DNA and then associate with the cell surface where it is internalised through an endocytotic process [43, 44]. During endosomal acidification, the histidine residues in the peptide become protonated and the peptide is able to interact with the membrane in its more active in-plane orientation (Fig. **2**). The resulting destabilisation of the lipids in the endosomal membrane leads to the release of the peptide/DNA complex into the cytosol where the plasmid DNA is presumably trafficked to the nucleus for expression. Clearly then with a complex process such as this there are many stages that could be optimised but one that stands out is the interaction of the vector peptide with the membrane. An endosome release mechanism for DOTAP/DNA complexes involving anionic lipid flip-flop has already been proposed [45] and hence we were interested to test for a role for anionic lipids in the transfection process mediated by LAH4. If anionic lipids are indeed required for release of the peptide/DNA complex then there may also be certain cell types where a surface presentation of anionic lipids, as is the case for certain transformed cells [46], could enhance the transfection efficiency mediated by a cationic peptide. Since the angle subtended by the charged residues has been shown to be a key factor in determining activity and selectivity for a number of antimicrobial or antitumour peptides [47], and suggested to alter the selectivity of the peptide for anionic over zwitterionic lipids, we designed LAH4 isomers where the angle subtended by the histidine residues, charged at acidic pH, was varied (Fig. **4A**) (Table **2**). We then screened the peptides using our ^{2}H solid-state NMR method, replacing PG with phosphatidylserine (PS) as the deuterated reporter lipid and including phosphatidylcholine (PC) as the zwitterionic lipid in place of PE, as well as cholesterol, to better replicate the target eukaryotic

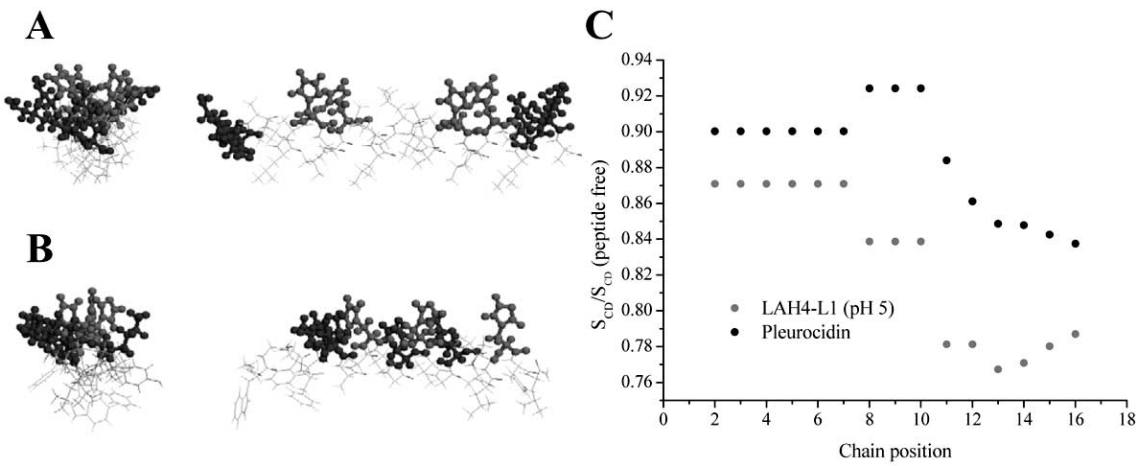

Fig. (3). Structural models comparing the antimicrobial peptides LAH4-L1 (**A**) and pleurocidin (**B**) in their active, in-plane orientations showing both end and side views. The lysine and histidine residues of each peptide are marked as ball and stick. The lysine residues of LAH4-L1 are sequestered at the peptide termini whilst those of pleurocidin are in the centre of the peptide and may prevent the histidine residues from interacting directly with the anionic lipid headgroups. A comparison of the order parameter profiles (**C**) of POPG-d31 in POPE:POPG (3:1) liposomes prepared in the presence of 2.5 mole% of each of these peptides reveals that LAH4-L1, in its active conformation (at acidic pH), destabilises the lipid chains far more effectively than pleurocidin. The profiles are calculated relative to peptide free liposomes and show the proportionally greater disruption in the lower half of the lipid, i.e. the hydrophobic core of the membrane.

endosomal membrane [8]. We also tested the *in vitro* transfection capabilities against four different human cell lines including three transformed cell lines and one primary human lung fibroblast cell line, MRC-5. Again we were able to show, using both the ^{2}H and also a ^{31}P MAS solid-state NMR method, that LAH4 interacts preferentially with the anionic lipid component of the mixed model membrane but here we were able to extend our study by being able to measure the membrane disruptive effect of the LAH4 isomers and distinguish between peptides with weaker or stronger disordering capabilities (Fig. **4**). We found that peptides with intermediate angles subtended by the four histidine residues (80° or 100°) were more disordering than peptides with either a very low (60°) or very high (180°) angle (Fig. **4B**). Interestingly, the peptides that were more effective at disordering the anionic lipids were also the most efficient vector peptides with LAH4-L1 (80°) and LAH4-L2 (100°) far more effective than the LAH4-L0 (60°) or LAH4-AL6 (180°) isomers (Fig. **4C**). Whilst with the study of the antimicrobial activity of LAH4 we had an indication that enhanced membrane disordering lead to more effective antibiotic activity, here we were able to relate the membrane disordering effect to the transfection efficiency by comparing relative indices of normalised membrane disordering and transfection efficiency [8] which showed that the model membrane system was directly relevant to the *in vitro* transfection activity (Fig. **4D**). In addition to this crucial finding, with its significance for using the model system for future peptide screens, was the finding that one peptide, LAH4-L1, was significantly more efficient as a vector peptide than any of its predecessors. In addition it was noted that because of the differing transfection efficiencies of the four peptides against either primary or transformed cell lines, it could be possible to select peptides that would remain efficient vector peptides against transformed tissue but would deliver hardly any DNA to primary tissue thus affording a selective delivery capacity to diseased tissues.

Here, the application of solid-state NMR is capable of providing quantitative information regarding the effect of varying peptide structure on membrane disordering and can be employed on a day to day basis as an effective screen of peptide properties which are directly related to their activity. Further improvements in peptide mediated transfection have been made possible by focussing on the membrane disruptive capabilities of new generation peptides and we have recently shown that peptides with increased histidine content are even stronger membrane destabilising agents and have proven to be robust transfection agents even in the presence of elevated levels of serum which commonly reduces non-viral mediated DNA transfer [10].

PERSPECTIVES

The application of ^{2}H solid-state NMR to the study of perdeuterated lipids has proven to be a valuable tool in relating structural features of membrane active peptides to their relative activities. It is likely that this system will prove amenable to the study of the influence of a wide range of membrane components on the membrane activity of candidate peptides and we envisage extending our studies to include more varied phospholipids and sterols to provide model membranes that better reflect the various target membranes from nature. Replacing two glycine residues in pleurocidin with alanine induces a dramatic increase in the activity of the peptide against zwitterionic PC membranes [48] and hence many other structural features affect the membrane interaction of antimicrobial or vector peptides and could be studied as described here.

Finally, whilst we have described a role for ^{2}H solid-state NMR in rational peptide design, advances in vector and antibiotic peptide technology may ultimately come from other sources. For a peptide of 26 residues in length such as LAH4, there are 26^{20} possible combinations of only the standard L-amino acids and hence the possibilities for improving activity are immense. The emerging technique of directed evolution by *in vitro* compartmentalisation [49] might be one method of screening the huge random peptide library that could be generated and has the potential to provide a quantum leap improvement in activity [50] and biophysical techniques, in particular those described here, seem well placed to interpret such advances.

CONCLUSION

Cationic amphipathic histidine rich peptides have both DNA vector and antimicrobial activity. Since the interaction between the peptide and biological membranes plays a crucial role in both activities, an understanding of the processes involved in membrane binding, disruption or translocation is important for the development of peptides with biotechnological or medicinal potential. We have used biophysical methods, predominantly solid-state NMR, to describe the specific peptide-membrane interactions and have related our findings to the biological activity of the peptides as both vectors and antimicrobial agents. We have used the information

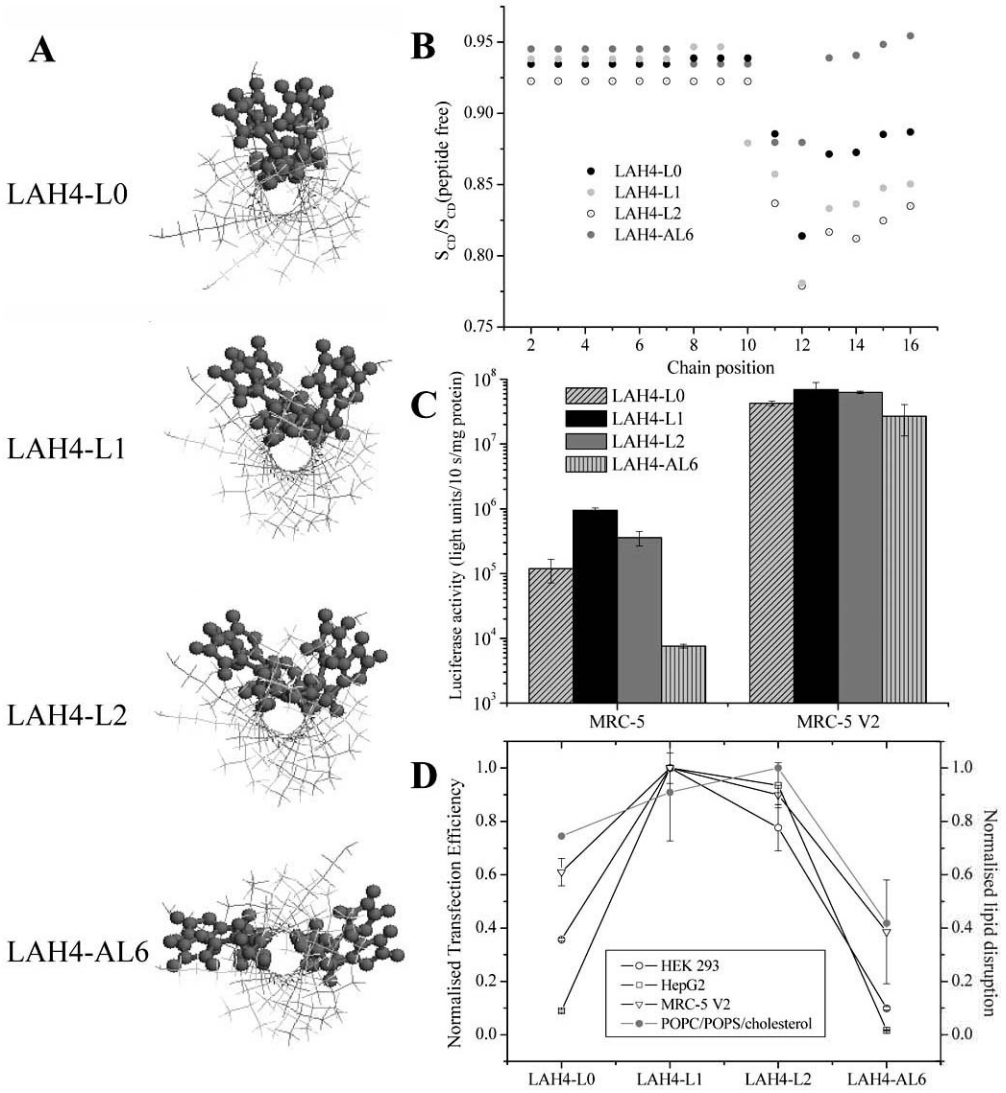

Fig. (4). Helical wheel diagrams for LAH4 isomers (**A**). The angles subtended by the positively charged helix face, at pH 5, for the peptides are 60° (L0), 80° (L1), 100° (L2) and 180° (AL6). ^{2}H order parameter profiles for peptide/POPS-d31 containing vesicles, calculated relative to POPS-d31 in peptide free vesicles (**B**) and a comparison of the relative transfection efficiency of the LAH4 vectors on two eukaryote cell lines (**C**). In the transfection experiments, increasing amounts of peptide were mixed with a constant amount of a plasmid encoding the *luciferase* gene. 30 hours post-transfection, cells were harvested, lysed and the luciferase activity was measured. Here only the formulation giving the highest luciferase activity is shown. Error bars represent the standard deviation of the mean. The relationship between the transfection efficiency and membrane disruption is shown (**D**) by comparing normalised transfection efficiencies for three different transformed cell lines and normalised lipid disruption efficiencies for the POPC/POPS/cholesterol model membranes calculated for an average of the lowest five segments of the POPS-d31 acyl chain. Figure adapted from [8].

obtained from our biophysical studies to design peptides that demonstrate improved efficiency in either domain. This review thus illustrates how biophysical methods such as solid-state NMR spectroscopy, by providing a better understanding of the peptide-membrane interactions, can aid the design of improved pharmaceuticals and biotechnological tools.

REFERENCES

[1] Tycko, R. *Protein Pept. Lett.*, **2006**, *13*, 229.

[2] Tycko, R. *Annu. Rev. Phys. Chem.*, **2001**, *52*, 575.

[3] Jakobsen, L.O.; Malmendal, A.; Nielsen, N.C.; Esmann, M. *Biochemistry*, **2006**, *45*, 10768.

[4] a) Watts, A. *Mol. Membr. Biol.*, ***2002***, *19*, 267. b) Watts, A. *Nat. Rev. Drug Discov.*, **2005**, *4*, 555.

[5] a) Williamson, P.T.; Watts, J.A.; Addona, G.H.; Miller, K.W.; Watts, A. *Proc. Natl. Acad. Sci. USA,* **2001**, *98*, 2346. b) Watts, J.A.; Watts, A.; Middleton, D.A. *J. Biol. Chem.*, **2001**, *276*, 43197. c) Middleton, D.A.; Rankin, S.; Esmann, M.; Watts, A. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 13602. d) Taylor, A.M.; Gröbner, G.; Williamson, P.T.F.; Watts. A. *Biochemistry*, **1999**, *38*, 11172.

[6] Williamson, P.T.; Bains, S.; Chung, C.; Cooke, R.; Watts, A. *FEBS Lett*., **2002**, *518,* 111.

[7] a) Luca, S.; Heise, H.; Lange, A.; Baldus, M. *Arch Pharm.*, **2005**, *338*, 217. b) Lange, A.; Giller, K.; Hornig, S.; Martin-Eauclaire, M-F.; Pongs, O.; Becker, S.; Baldus, M. *Nature*, **2006**, *440*, 959.

[8] Mason, A.J.; Martinez, A.; Glaubitz, C.; Danos, O.; Kichler, A.; Bechinger, B. *FASEB J.,* **2006**, *20*, 320.

[9] Mason, A.J.; Gasnier, C.; Kichler, A.; Prévost, G.; Aunis, D.; Metz-Boutigue, M.-H.; Bechinger, B. *Antimicrob. Agents Chemother.*, **2006**, *50*, 3305.

[10] Mason, A.J.; Leborgne, C.; Moulay, G.; Martinez, A.; Danos, O.; Bechinger, B; Kichler, A. *J. Control. Release*, **2007**, *118*, 95.

[11] Watts, A. *Biochim. Biophys. Acta*, **1998**, *1376*, 297.

[12] a) Seelig, J. *Biochim. Biophys. Acta* **1978**, *515*, 105. b) Bechinger, B. *Biochim. Biophys. Acta*, **2005**, *1712*, 101.

[13] a) Sixl, F.; Watts, A. *Proc. Natl. Acad. Sci. USA*, ***1983***, *80*, 1613. b) Seelig, J.; Macdonald, P.M.; Scherer, P.G. *Biochemistry*, **1987**, *26*, 7535. c) Scherer, P.G.; Seelig, J. *Biochemistry*, **1989**, *28*, 7720. d) Roux, M.; Neumann, J.M.;

Hodges, R.S.; Devaux, P.F.; Bloom, M. *Biochemistry*, **1989**, *28*, 2313. e) Bechinger, B.; Seelig, J. *Biochemistry*, **1991**, *30*, 3923.

[14] a) Pinheiro, T.J.T.; Watts, A. *Biochemistry*, **1994**, *33*, 2459. b) Carbone, M.A.; Macdonald, P.M. *Biochemistry*, **1996**, *35*, 3368. c) Bonev, B.B.; Chan, W.C.; Bycroft, B.W.; Roberts, G.C.K.; Watts, A. *Biochemistry*, **2000**, *39*, 11425. d) Bonev, B.; Watts, A.; Bokvist, M.; Gröbner, G. *Phys. Chem. Chem. Phys*., **2001**, *3*, 2904.

[15] a) Siminovitch, D.J.; Brown, M.F.; Jeffrey, K.R. *Biochemistry,* **1984**, *23*, 2412. b) Lindstrom, F.; Williamson, P.T.; Grobner, G. *J. Am. Chem. Soc.,* **2005**, *127*, 6610.

[16] a) Huster, D.; Arnold, K.; Gawrisch, K. *J. Phys. Chem. B,* **1999**, *103*, 243. b) Huster, D.; Vogel, A.; Katzka, C.; Scheidt, H.A.; Binder, H.; Dante, S.; Gutberlet, T.; Zschornig, O.; Waldmann, H.; Arnold, K. *J. Am. Chem. Soc*., **2003**, *125*, 4070. c) Siarheyeva, A.; Lopez, J.J.; Glaubitz, C. *Biochemistry,* **2006**, *45*, 6203. d) Gallagher, G.J.; Hong, M.; Thompson, L.K. *Biochemistry*, **2004**, *43*, 7899.

[17] a) Mayer, C.; Muller, K.; Weisz, K.; Kothe, G. *Liq. Cryst.,* **1988**, *3*, 797. b) Mayer, C.; Grobner, G.; Muller, K.; Weisz, K.; Kothe, G. *Chem. Phys. Lett.,* **1990**, *165*, 155. c) Pinheiro, T.J.T.; Duer, M.J.; Watts, A. *Solid State Nucl. Magn. Reson.,* **1997**, *8*, 55. d) Dave, P.C.; Tiburu, E.K.; Damodaran, K.; Lorigan, G.A. *Biophys. J.,* **2004**, *86*, 1564. e) Lu, J.X.; Damodaran, K.; Blazyk, J.; Lorigan, G.A. *Biochemistry,* **2005**, *44*, 10208.

[18] a) Hong, M.; Schmidt-Rohr, K.; Nanz, D. *Biophys. J.,* **1995**, *69*, 1939. b) Soubias, O.; Reat, V.; Saurel, O.; Milon, A. *J. Magn. Reson.,* **2002**, *158*, 143. c) Middleton, D.A.; Hughes, E.; Madine, J. *J. Am. Chem. Soc.,* **2004**, *126*, 9478. d) Lu, J.X.; Damodaran, K.; Lorigan, G.A. *J. Magn. Reson.,* **2006**, *178*, 283.

[19] a) Seelig, J.; Niederberger, W. *J. Am. Chem. Soc*., **1974**, *96*, 2069. b) Davis, J.H. *Biochim. Biophys. Acta*, **1983**, *737*, 117.

[20] www.avantilipids.com

[21] a) Seelig, A.; Seelig, J. *Biochemistry,* **1974**, *13*, 4839.

[22] Prosser, R.S.; Hwang, J.S.; Vold, R.R. *Biophys. J*., **1998**, *74*, 2405.

[23] Mason, A.J.; Lopez, J.J.; Beyermann, M.; Glaubitz, C. *Biochim. Biophys. Acta*, **2005**, *1714*, 1.

[24] a) Sternin, E.; Bloom, M.; MacKay, A.L. *J. Magn. Reson*., **1983**, *55*, 274. b) Schäfer, H.; Mädler, B.; Volke, F. *J. Magn. Reson*., **1995**, *116*, 145.

[25] Roux, M.; Beswick, V.; Coïc, Y-M.; Huynh-Dinh, T.; Sanson, A.; Neumann, J-M. *Biophys. J*., **2000**, *79*, 2624.

[26] Urbina, J.A.; Pekerar, S.; Le, H-b.; Patterson, J.; Montez, B.; Oldfield, O. *Biochim. Biophys. Acta*, **1995**, *238*, 163.

[27] Dave, P.C.; Tiburu, E.K.; Nusair, N.A.; Lorigan, G.A. *Solid State Nucl. Magn. Reson*., **2003**, *24*, 137.

[28] Mason, A.J.; Chotimah, I.N.; Bertani, P.; Bechinger, B. *Mol. Membr. Biol*., **2006**, *23*, 185.

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[29] Bechinger, B. *J. Mol. Biol*., **1996**, *263*, 768.

[30] Vogt, T.C.B.; Bechinger, B. *J. Biol. Chem*., **1999**, *274*, 29115.

[31] Cole, A.M.; Weis, P.; Diamond, G. *J. Biol. Chem*., **1997**, *272*, 12008.

[32] Patrzykat, A.; Douglas, S.E. *Trends Biotechnol*., **2003**, *21*, 362.

[33] Patrzykat, A.; Gallant, J.W.; Seo, J.K.; Pytyck, J.; Douglas, S.E. *Antimicrob. Agents Chemother.*, **2003**, *47*, 2464.

[34] Syvitski, R.T; Burton, I.; Mattatall, N.R; Douglas, S.E; Jakeman, D.L. *Biochemistry*, **2005**, *44*, 7282.

[35] Yoshida, K.; Mukai, Y.; Niidome, T.; Takashi, C; Tokunaga, Y.; Hatakeyama, T; Aoyagi, H. *J. Peptide Res*., **2001**, 57, 119.

[36] Saint, N.; Cadiou, H.; Bessin, Y.; Molle, G. *Biochim. Biophys. Acta*, **2002**, *1564*, 359.

[37] Patrzykat, A.; Friedrich, C.L.; Zhang, L.; Mendoza, V.; Hancock, R.E.W. *Antimicrob. Agents Chemother.*, **2002**, *46*, 605.

[38] Brogden, K.A. *Nat. Rev. Microbiol*., **2005**, *3*, 238.

[39] a) Lauth, X.; Shike, H.; Burns, J.C.; Westerman, M.E.; Ostland, V.E.; Carlberg, J.M.; Van Olst, J.C.; Nizet, V.; Taylor, S.W.; Shimizu, C.; Bulet, P. *J. Biol. Chem*., **2002**, *277*, 5030. b) Silphaduang, U.; Noga, E.J. *Nature*, **2001**, *414*, 268.

[40] Iijima, N.; Tanimoto, N.; Emoto, Y.; Morita, Y.; Uematsu, K.; Murakami, T.; Nakai, T. *Eur. J. Biochem*., **2003**, *270*, 675.

[41] Cole, A.M.; Darouiche, R.O.; Legarda, D.; Connell, N.; Diamond, G. *Antimicrob. Agents Chemother.*, **2000**, *44*, 2039.

[42] Ramamoorthy, A.; Thennarasu, S.; Lee, D-K.; Tan, A.; Maloy, L. *Biophys. J*., **2006**, *91*, 206.

[43] Kichler, A.; Leborgne, C.; März, J.; Danos, O.; Bechinger, B. *Proc. Natl. Acad. Sci. USA*, **2003**, *96*, 1564.

[44] Kichler, A.; Mason, A.J.; Bechinger, B. *Biochim. Biophys. Acta*, **2006**, *1758*, 301.

[45] Zelphati, O,; Szoka, F.C.; Jr., *Proc. Natl. Acad. Sci. USA*, **1996**, *93*, 11493.

[46] Utsugi, T.; Schroit, A.J.; Connor, J.; Bucana, C.D; Fidler, I.J. *Cancer Res*., **1991**, *51*, 3062.

[47] a) Wieprecht, T.; Dathe, M.; Epand, R.M.; Beyermann, M.; Krause, E.; Maloy, W.L.; MacDonald, D.L.; Bienert, M. *Biochemistry*, **1997**, *36*, 12869. b) Yang, N.; Stensen, W.; Svendsen, J.S.; Rekdal, Ø. *J. Peptide Res*., **2002**, *60*, 187.

[48] Yang, J.Y.; Shin S.Y.; Lim, S.S.; Hahm, K-S.; Kim, Y. *J. Microbiol. Biotechnol*., **2006**, *16*, 880.

[49] Miller, O.J.; Bernath, K.; Agresti, J.J.; Amitai, G.; Kelly, B.T.; Mastrobattista, E.; Taly, V.; Magdassi, S.; Tawfik, D.S.; Griffiths, A.D. *Nat. Methods*, **2006**, *3*, 561.

[50] Griffiths, A.D.; Tawfik, D.S. *EMBO J*., **2003**, *22*, 24.