The Gramicidin S structural motif: a promising platform for the rational design of antibiotic agents

Ph.D. thesis

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2005

Contents

Abbreviations

AMPs	antimicrobial peptides
CD	circular dichroism
DE-DGDG	dielaidoyl diglucosyldiacylglycerol
DE-MGDG	dielaidoyl monoglucosyldiacylglycerol
DEPC	dielaidoylphosphatidylcholine
DMPC	dimyristoylphosphatidylcholine
DMPE	dimyristoylphosphatidylethanolamine
DMPG	dimyristoylphosphatidylglycerol
DMPS	dimyristoylphosphatidylserine
DSC	differential scanning calorimetry
ESR	electron spin resonance
FTIR	Fourier transform infrared
GS	gramicidin S, cyclo [VOLFPVOLFP]
GS10	gramicidin S 10, cyclo[VKLYPVKLYP]
GS12	gramicidin S 12, cyclo[VKLKYPKVKLYP]
GS14	gramicidin S 14, cyclo [VKLKVYPLKVKLYP]
GS14*d*K$_4$	*d*Lys (position 4) substituted gramicidin S 14, cyclo[VKLKVYPLKVKLYP]
L_α	lamellar liquid-crystalline phase
L_β or $L_{\beta'}$	lamellar gel phase with untilted or tilted hydrocarbon chains
L_C or $L_{C'}$	lamellar crystalline phase with untilted or tilted hydrocarbon chains
LD_{50}	concentration required for 50% loss of integrity
Leu	leucine
LPS	lipopolysaccharide
LUV	large unilamellar vesicles
Lys	lysine
MLV	multilamellar vesicles
NMR	nuclear magnetic resonance
Orn	ornithine
$P_{\beta'}$	lamellar rippled gel phase with tilted hydrocarbon chains
Phe	phenylalanine
P-O-Et-DMPC	P-O-ethyl dimyristoylphosphatidylcholine
POPC	1-palmitoyl-2-oleoylphosphatidylcholine
POPE	1-palmitoyl-2-oleoylphosphatidylethanolamine
POPG	1-palmitoyl-2-oleoylphosphatidylglycerol
Pro	proline
RP-HPLC	reversed phase high performance liquid chromatography
14-SASL	(4,4-dimethyloxazolidine-N-oxyl)-stearic acid spin label
SDS	sodium dodecyl sulfate
τ_{eff}	effective rotational correlation time
TFE	trifluoroethanol
T_m	hydrocarbon chain melting phase transition temperature
TMCL	tetramyristoylcardiolipin
Tyr	tyrosine
Val	valine

List of publications

Full papers directly related to the subject of the thesis:

I. **Kiricsi M.**, Horváth L.I., Dux L., Páli T. (2001) Spin label EPR studies of the effect of gramicidin S on lipid chain dynamics *J MOL STRUCT* **563(SI)**:469-475 **(IF: 1.122)**

II. **Kiricsi M.**, Prenner E.J., Jelokhani-Niaraki M., Lewis R.N.A.H., Hodges R.S., McElhaney R.N. (2002) The effects of ring-size analogs of the antimicrobial peptide gramicidin S on phospholipid bilayer model membranes and on the growth of *Acholeplasma laidlawii* B *EUR J BIOCHEM* **269**:5911-5920 **(IF: 2.999)**

III. Lewis R.N.A.H., **Kiricsi M.**, Prenner E.J., Hodges R.S., McElhaney R.N. (2003) Fourier transform infrared spectroscopic study of the interactions of a strongly antimicrobial but weakly hemolytic analogue of gramicidin S with lipid micelles and lipid bilayer membranes *BIOCHEMISTRY-US* **42**:440-449 **(IF: 4.064)**

IV. Prenner E.J., **Kiricsi M.**, Jelokhani-Niaraki M., Lewis R.N.A.H., Hodges R.S., McElhaney R.N. (2005) Structure-activity relationships of diastereomeric lysine ring-size analogs of the antimicrobial peptide gramicidin S: mechanism of action and discrimination between bacterial and animal cell membranes *J BIOL CHEM* **280:** 2002-2011 **(IF: 6.482)**

Other papers:

V. Putman C.T., Dixon W.T., Pearcey J.A., MacLean I.M., Jendral M.J., **Kiricsi M.**, Murdoch G.K. and Pette D. (2004) Chronic low-frequency stimulation up-regulates uncoupling protein-3 in transforming rat fast-twitch skeletal muscle *AM J PHYSIOL Regul Integr Comp Physiol* **287**:R1419-1426 **(IF: 3.627)**

VI. Putman C.T., **Kiricsi M.**, Pearcey J., MacLean I.M., Bamford J.A., Murdoch G.K., Dixon W.T., Pette D. (2003) AMPK activation increases UCP-3 expression and mitochondrial enzyme activities in rat muscle without fiber type transitions *J PHYSIOL* **551**: 169-78 **(IF: 4.650)**

VII. Horváth D., **Kiricsi M.**, Tóth Á. (1998) Lateral front instability in an open reaction-diffusion system *J CHEM SOC Faraday Trans* **94**: 1217-1219 **(IF: 1.757)**

VIII. **Kiricsi M.**, Berkesi O. (1997) Vesekövek osztályozása *A KÉMIA TANÍTÁSA* **(V)3-4**:3-6.

Summary

Significant scientific effort is focused on antimicrobial peptides worldwide because they might represent a new antibiotic class in the future. Their primary target is not a specific bacterial protein but the entire bilayer structure of the bacterial membrane. Because of this fact the probability of bacteria developing resistance against these substances is rather low, which greatly enhances the future potential of antimicrobial peptides and renders them attractive drug design candidates. Although the exact mechanism of action is currently being widely investigated for several types of antimicrobial peptides, one unique mechanism seems unlikely despite the fact that most of them act by degrading the integrity of the bacterial cell membrane. Therefore it is very important to find the basis of the membrane disruptive activity of a particular peptide structural motif and of its structural analogs. In our studies we focused on gramicidin S (GS), a natural cyclic antimicrobial peptide and on its synthetic structural analogs. Two series of structural variations of the GS molecule were examined in detail. The ring-size analogs of GS were constructed to have extended ring sizes (GS12 is composed of 12, GS14 of 14 amino acids) compared to the cyclodecapeptide GS. The second series consists of GS14 analogs with enantiomeric inversions of one or more of the four Lys residues present in the GS14 structure. It has been previously shown that a very promising peptide of this second series, GS14*d*K_4 exhibits preferential lytic capacity towards bacteria. Further studies were done to elucidate the molecular basis of its improved specificity. Mainly biophysical methods and some simple microbiological assays were performed. GS and its synthetic ring-size analogs (GS10, GS12 and GS14) were examined in their ability to perturb the characteristic phase transition behavior of various phospholipids using spin-label electron spin resonance (ESR) spectroscopy and differential scanning calorimetry. The synthetic ring size analogs broadened and shifted the phase transition temperature range of each phospholipid system, but were more effective at perturbing zwitterionic PC and anionic PG than zwitterionic PE phase transitions. The synthetic ring size analogs were tested also in that respect, how effectively are they able to perturb the integrity of model or biological phospholipid bilayers. We screened firstly their potency to induce the leakage of a fluorescent dye encapsulated inside vesicles composed of various phospholipids. Secondly their capacity to inhibit the proliferation of the cell wall-less mollicute *Acholeplasma laidlawii* B culture was assessed in order to examine the direct interaction between these peptides and their primary target the lipid bilayer of the bacterial membrane. The relative potency of the ring-size analog peptides to perturb model and biological bilayers generally decreased in the following order: GS14 > GS10 > GS12. In each assay GS14 was the

most potent of the ring-size analogs, which implies the advantage of a larger ring size, of the increased charge and amphiphilicity possessed by GS14 in membrane permeation. Somewhat surprising was the high toxicity of GS14 against *A. laidlawii*, which contrasts its weak activity against conventional bacteria. This finding suggests that its avid binding to bacterial cell wall components may be responsible for its low antimicrobial activity when measured against conventional bacterial cultures. The second part of the present thesis is based on the examination of the structure-activity relationships of GS14 analogs with enantiomeric inversions of its Lys residues. The structural characteristics of these peptides were examined by circular dichroism (CD) spectroscopy. Their capacities to induce dye leakage from phospholipid vesicles and to inhibit the growth of *A. laidlawii* B have also been assessed to investigate their potency to degrade membrane barrier function. These studies indicate that the peptides can be classified into 3 groups: 1. GS14, the most structured, the most amphiphilic and most effective membrane-permeating peptide. 2. The multiple-inversion analogs (GS14*d*K_2K_4, GS14*d*$K_2K_4K_9K_{11}$), the least structured and least effective as antimicrobial and membrane perturbing agents. 3. The single inversion analogs (GS14*d*K_2, GS14*d*K_4, GS14*d*K_9, GS14*d*K_{11}). As enantiomeric Lys inversions of GS14 are inequivalent, the single-inversion analogs each have unique antibiotic and membrane-disruptive properties. Moreover, although they are not as disruptive on membranes as GS14, they have greater capacity to discriminate between bacterial and animal cell membranes. One peptide in this series, GS14*d*K_4, has the greatest preferential lytic capacity towards bacterial membranes. Therefore it was examined further to elucidate the molecular basis of its improved specificity. Fourier transform infrared spectroscopic (FTIR) studies on the interaction between GS14*d*K_4 and various single- and multi-component lipid membranes were performed. These studies indicated that GS14*d*K_4 penetrated more deeply into single-component anionic than into zwitterionic or nonionic phospholipid membranes, and was largely excluded from cationic bilyers. Anionic lipids facilitated GS14*d*K_4 interactions with various multi-component lipid bilayers. The peptide did not penetrate/partition into cholesterol-containing PC membranes, which mimicked the composition of mammalian cell membranes, thus its apparent low solubility in these bilayers may form the basis of its reduced capacity to lyse erythrocytes. Its strong interactions with anionic lipids combined with its greater size and altered distribution of positive charges compared to GS make GS14*d*K_4 intrinsically more perturbing of membrane organization, which explains its high antimicrobial activity. These insights should assist in the design of potent, more selective and specific therapeutically useful GS-like antimicrobial peptides.

1. Introduction

1.1. Antimicrobial peptides

The past 50 years have been the "antibiotic era" in which natural, semisynthetic and synthetic antimicrobial agents have been used against life-threatening infections [1]. This era might end soon because of the emergence of pathogenic bacteria with clinically significant resistance to conventional antibacterial agents. This is already a major public health concern, and provides much of the impetus for current attempts to develop newer types of clinical antibiotics to evade bacterial drug resistance mechanisms [1, 2, 3]. Microbial resistance to antibacterial agents develops when a bacterial population produces mutants to counteract the effects of the antibiotic. This involves mutations of enzymes or receptors, which bind transport or metabolize the antibiotic. The mutations cause decreasing rates of entry, eventually degradation, inactivation or removal of the drug from the site of action [3]. Unfortunately, due to the genetic plasticity and short life cycles, antibiotic-resistant strains are likely to emerge far more rapidly than the time required for the development of new antibiotic classes. Over the past decades, the search for new drugs prompted a special interest in a group of short polypeptides, named antimicrobial peptides (AMPs). They are among the most promising research candidates and have been the focus of investigation in a number of laboratories worldwide. AMPs are particularly attractive because they are usually part of the innate immune response of higher organisms. The fact that huge arsenals of AMPs have been proven effective over evolutionary time is a good indicator of their potential usefulness. Although recent studies have shown that some of the peptides probably have intracellular targets, the great majority of AMPs appear to act by permeabilization of the bacterial cell membrane. This finding suggests a mode of action that involves a direct interaction of the peptides with cell membranes [4]. AMPs attack the membrane bilayer structure and their accumulation results in degradation of the barrier properties, in loss of the integrity and increased permeability of bacterial cell membranes. Eventually they cause local ruptures in the membrane where cellular components can leak out [5]. The fact that their target is not a specific protein or enzyme, as it is the case with conventional antibiotics, but the entire bilayer structure, might enhance their potential and avoid drug resistance. In the presence of antimicrobial peptides the microbe would have to re-design its membrane. Changing the composition and/or organization of its lipids is probably an expensive solution for most microbial species. Moreover, in the presence of their own protein constituents, selective destruction of antimicrobial peptides would be problematic as well [6]. Because of this non-specific mode of action, the development of bacterial resistance to these

agents is rather difficult. In fact most cationic peptides do not induce the appearance of specific mutant bacteria [1]. However we should note that there are a number of bacterial species that have already developed resistance to AMPs by modification of the bacterial cell envelope components or by employing outer membrane proteases or efflux pumps [4, 7].

Antimicrobial peptides were first discovered in plants, then in other organisms, including humans [4]. A great number of naturally occurring and newly designed peptides exhibit antimicrobial and antifungal activities. Unfortunately, many AMPs are not suitable for general clinical application, mainly because of their tendency to cause hemolysis of mammalian cells. Consequently, much effort is currently being expended to increase the potency and the specificity of such peptides aiming to produce antimicrobial peptides of comparable antibiotic effectiveness but with a reduced toxicity to animal cells [5]. These peptides are generally 10-50-residue polypeptides, and most of them possess a net positive charge. AMPs are usually highly amphipathic molecules, which property allows them to bind to the membrane interface [5]. Linear peptides can form amphipathic and hydrophobic helices, cyclic peptides possess β–sheet structures or loop structures stabilized by disulfide bonds. Given the wide variety of structural and conformational motifs exhibited by membrane-targeted antimicrobial peptides, it is probably unlikely that they all disrupt membranes by a similar mechanism. This is an important concept to establish, because it means that one should not be thinking of a global mechanism of action but rather of what is the basis of the membrane disruptive activity of a particular structural/conformational motif. However structurally related families of antimicrobial peptides will probably disrupt cell membranes by similar mechanisms.

1.2. Gramicidin S

Gramicidin S (GS) is a cyclic decapeptide of the primary structure [cyclo-(Val-Orn-Leu-*d*Phe-Pro)$_2$]. The peptide was first isolated from *Bacillus brevis* [8] and is one of a series of antimicrobial peptides produced by this microorganism [9, 10]. Structural studies have shown that GS forms an amphipathic, two-stranded, antiparallel β-sheet, in which hydrophobic residues (Val, Leu) reside on one face of the molecule and basic residues (Orn) are located on the opposite face [11]. The antiparallel β-sheet is linked on each side by a type II' β-turn formed by the two *d*Phe-Pro sequences. Two intramolecular hydrogen bonds, involving the amide protons and carbonyl groups of the two Leu and two Val residues, stabilize the structure (Figure 1). This conformation of the GS molecule is maintained in water, in organic solvents of varying polarity, in detergent micelles and phospholipid bilayers, even at high temperatures and in the presence of agents, which often alter protein conformation [12].

GS shows potent antibiotic activity against a broad spectrum of Gram-negative and Gram-positive bacteria and several pathogenic fungi. It is also strongly hemolytic. This feature limits its therapeutic utilization to topical applications [9, 10]. GS interacts strongly with membrane lipid components. These interactions involve hydrophobic interactions between the membrane interface and the hydrophobic surface of the peptide. Electrostatic interactions are formed between the basic residues of the peptide and the polar head group of anionic lipids, which are abundant in bacterial membranes. For this reason, the number and the relative positioning of hydrophobic and basic residues in the peptides are essential for the antibiotic activity [11].

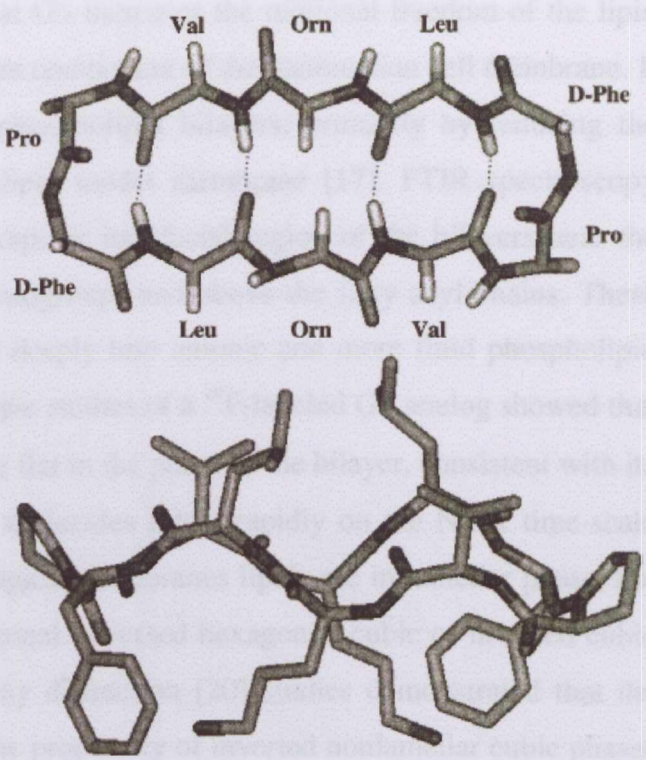

Figure 1. Structure and conformation of gramicidin S. Upper panel shows top view of the peptide backbone with the dashed lines representing the possible cross-ring hydrogen bonding, whereas the lower panel shows side view of the peptide backbone and the orientations of the side chains relative to the ring plane.

In the last decade, GS has been thoroughly investigated, thus its physico-chemical and biological characteristics are now fairly well understood. Its effect on phospholipid bilayers and on biological membranes has been examined in detail with various experimental techniques among those DSC. These studies indicated that GS interacts with various phospholipid bilayers. Its interaction with anionic bilayers is stronger than with zwitterionic phospholipid bilayers and stronger with model membrane systems of higher fluidity [13]. These results contradicted the findings of two earlier studies by Pache [14] and Finer [15] using ESR and nuclear magnetic resonance (NMR) spectroscopy. They investigated the effect of GS addition on the mobility of 12-nitroxide methylstearate spin label in lecithin-water model membranes and on egg PC vesicles. They found no significant effect of GS addition and concluded that GS interacts only with the polar region of the bilayer. However, their data indicate some interaction between GS and the bilayer when carefully inspected [12]. Densitometry and sound velocimetry experiments showed that GS binding to PC bilayers decreases the temperature and cooperativity of their gel/liquid-crystalline phase transition. GS binding increases the volume compressibility and decreases the

density of the host bilayer [16], indicating that GS increases the motional freedom of the lipid hydrocarbon chains. Cholesterol is an important component of the mammalian cell membrane. It attenuates the disordering effect of GS on phospholipid bilayers, primarily by reducing the penetration of the peptide into the phospholipid model membrane [17]. FTIR spectroscopy demonstrated that GS is located at the polar/apolar interfacial region of the bilayers near the glycerol backbone region, below the polar headgroups and above the fatty acyl chains. These studies showed also that GS penetrates more deeply into anionic and more fluid phospholipid bilayers [18]. Solid-state ^{19}F-NMR spectroscopic studies of a ^{19}F-labeled GS analog showed that GS is aligned with its cyclic β-sheet ring lying flat in the plane of the bilayer, consistent with its amphiphilic character. However, the peptide molecules rotate rapidly on the NMR time-scale and wobble in fluid PC bilayers [19]. In biological membranes lipids are in lamellar phase, but they can assume non-lamellar micellar, hexagonal, reversed hexagonal, cubic or inverted cubic phases. ^{31}P-NMR spectroscopy [11] and X-ray diffraction [20] studies demonstrated that the insertion of GS into the bilayer can increase the propensity of inverted nonlamellar cubic phases in various phospholipid vesicles by producing negative monolayer curvature stress. It has been shown as well that GS causes thinning of the bilayers [11, 20].

1.3. Structural analogs of gramicidin S

Unfortunately, because of the non-specific mode of its action GS is also strongly toxic to red blood cells, which despite of its high antimicrobial activity, limits its use as a clinical antibiotic to topical applications [21]. However, recent studies showed that structural analogs of GS can be designed with dissociated antimicrobial and hemolytic activities [22]. The possibility of separately modifying the above described characteristics together with some of the key features of the GS molecule made it an attractive drug design candidate. These features were: 1. its rigid defined β–sheet structure, which is suitable for structure-activity studies, as the differences in structure and activity of the analogs can easily be compared to the parent molecule, 2. its small size allows easy synthesis, 3. the cyclic nature of the structure makes it resistant to proteolysis, which is an important issue of drug stability [23]. Thus the structural framework of GS was taken as a platform for rational and systematic design of cyclic membrane-active cationic peptides. The appropriate derivative(s) would have increased specificity for prokaryotic (bacterial and fungal) cells over eukaryotic membranes, consequently the ideal peptide(s) could be used in the future as potent oral or injectable broad-spectrum antibiotics [12, 21, 22, 23]. The relationship between the chemical structure of GS analogs and their hemolytic and antibiotic activities has been the subject of numerous studies [9, 10].

Unfortunately, most structural modifications of the GS molecule itself result in more or less parallel variations of biological activities against bacterial and animal cells. However, by varying both the size of the GS ring system and the orientation of certain amino acid side chains relative to the plain of the ring, one can obtain analogs which exhibit considerable (>100 fold) dissociation of their antimicrobial activity from their hemolytic properties [21, 22, 24, 25]. In fact, the more promising of such analogs (GS14*d*K$_4$) possess a therapeutic index, which is especially improved compared to the parent molecules and exhibit enhanced capacity to discriminate between bacterial and animal cell membranes [26, 27].

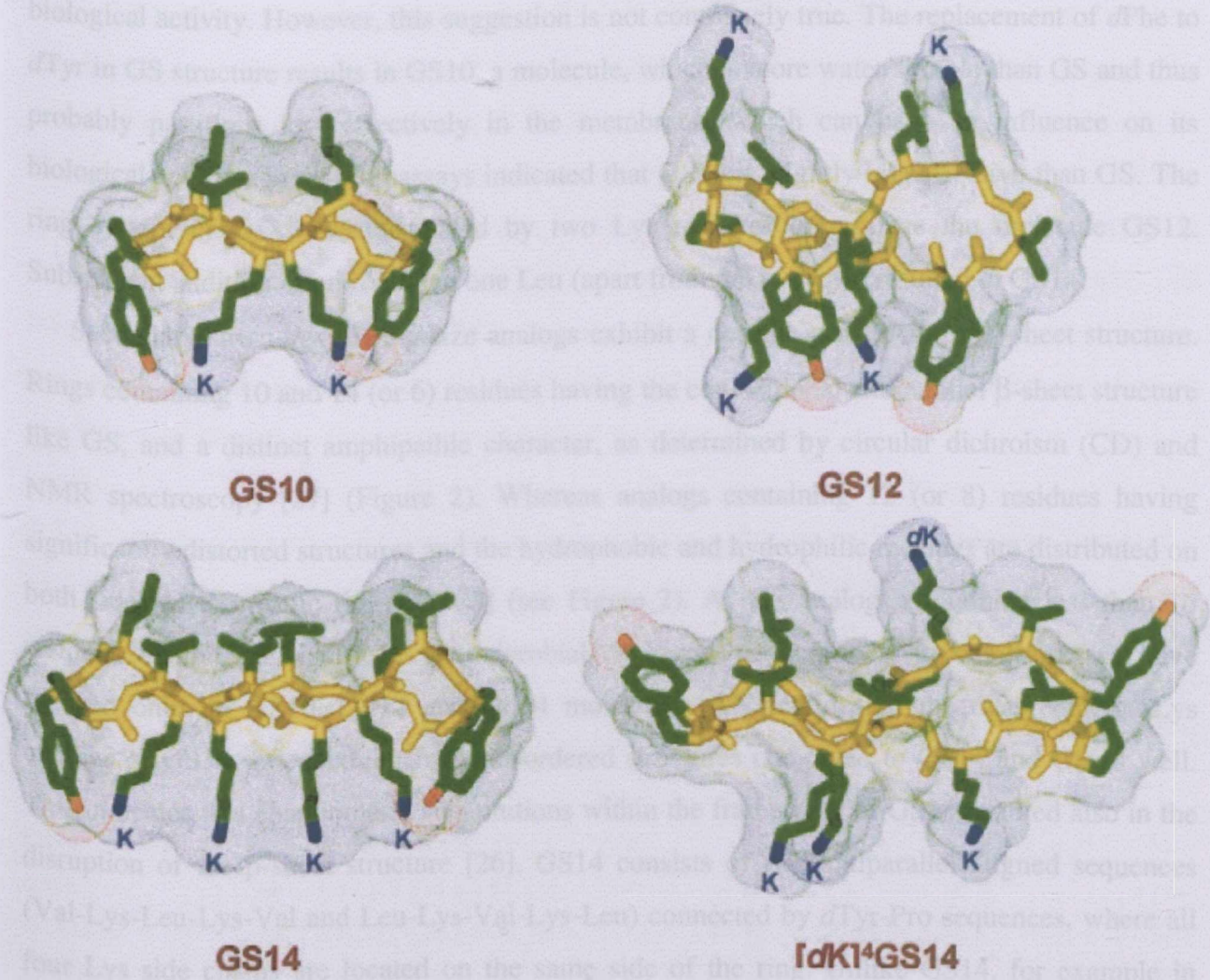

Figure 2. Stucture and conformation of four GS analogs in aqueous solution. Side views of the peptide backbone (yellow) and orientations of the side chains (green) are shown in this figure. Lys residues are marked with the letter K, hydroxyl groups are colored with red, amino groups with blue. Upper left panel shows GS10 (cyclo[VKLYPVKLYP]), upper right panel GS12 (cyclo[VKLKYPKVKLYP]) bottom left panel GS14 (cyclo [VKLKVYPLKVKLYP]) and bottom right panel indicates the most promising analog GS14*d*K$_4$ (cyclo [VKLKVYPLKVKLYP]) from the series of D-Lys substituted GS14 based analogs.

Thus the focus of our studies shifted to structural variations of GS, including variations in ring size (GS10, GS12 and GS14) and to a series of GS14 based analogs with enantiomeric inversions of one or more of the four Lys residues (GS14*d*K_2, GS14*d*K_4, GS14*d*K_9, GS14*d*K_{11}, GS14*d*K_2K_4, GS14*d*$K_2K_4K_9K_{11}$) in particularly to GS14*d*K_4 (Figure 2.) [22, 24, 25, 26]. The peptide structures are all based on the structure of GS except for two conservative amino acid substitutions. The Orn residues were replaced by Lys and the *d*Phe with *d*Tyr residues. It has been suggested that these conservative replacements increased only the water solubility and greatly reduced the costs of peptide synthesis, but did not by themselves alter conformation or biological activity. However, this suggestion is not completely true. The replacement of *d*Phe to *d*Tyr in GS structure results in GS10, a molecule, which is more water soluble than GS and thus probably partitions less effectively in the membrane, which can have an influence on its biological activity. In fact, bioassays indicated that GS10 is slightly less effective than GS. The ring system of GS10 was extended by two Lys residues to produce the molecule GS12. Subsequent addition of one Val and one Leu (apart from the two Lys) resulted in GS14.

Secondary structures of ring-size analogs exhibit a definite periodicity in β-sheet structure. Rings containing 10 and 14 (or 6) residues having the conventional antiparallel β-sheet structure like GS, and a distinct amphipathic character, as determined by circular dichroism (CD) and NMR spectroscopy [27] (Figure 2). Whereas analogs containing 12 (or 8) residues having significantly distorted structures and the hydrophobic and hydrophilic residues are distributed on both sides of the cyclic ring [22, 28] (see Figure 2). As GS analogs containing less than 10 residues exhibit no significant antimicrobial or hemolytic activities, in our work we have focused only on GS10, GS12 and GS14 molecules. All peptides of the distereomeric Lys analogs of GS14 series exhibit more disordered structures compared to GS14 and GS as well. This indicates that enantiomeric substitutions within the framework of GS14 resulted also in the disruption of the β-sheet structure [26]. GS14 consists of two antiparallel aligned sequences (Val-Lys-Leu-Lys-Val and Leu-Lys-Val-Lys-Leu) connected by *d*Tyr-Pro sequences, where all four Lys side chains are located on the same side of the ring. Unlike GS14, for example in GS14*d*K_4 the Lys-4 side chain projects to the otherwise completely hydrophobic face of the molecule thus diminishing its amphipathic character. Moreover, CD and ^{1}H-NMR spectroscopic studies have both shown, that GS14*d*K_4 adopts a distorted β-sheet conformation, that is less rigid, and considerably more sensitive to the polarity of the environment than GS14, presumably because of the disruption of the cross-ring H-bonding by the enantiomeric substitution [24, 27]. The peptide is also less amphipathic, more water soluble and less inclined to aggregate in aqueous solution or to interact strongly with hydrophobic surfaces than is GS14 [24].

As their structures so the biological activity of the peptides vary greatly. GS10 has comparable antimicrobial and hemolytic activity as GS [22]. The 12-residue peptide retains moderate activity against Gram-negative bacteria and fungi and exhibits reduced activity against Gram-positive bacteria. Most importantly, GS12 displays a significantly reduced hemolytic activity [22]. The therapeutic index is defined as the ratio of antimicrobial activity/ hemolytic activity resulting in a unitless value. So the decreased hemolytic activity of GS12 is reflected in its improved therapeutic index and specificity for Gram-negative bacteria. GS14 seems to be the least effective peptide, as it shows greatly reduced antimicrobial activity against bacteria but increased hemolytic activity compared to GS [22]. The antibiotic and hemolytic activities and the capacity to discriminate between bacterial and animal cells span a fairly broad range in the series of diastereomeric Lys analogs of GS14 as well [26]. GS14*d*K_4 is the most promising in this second analog series. It is comparably potent in its antibacterial and antifungal activity like GS, however is 15-20 fold less hemolytic. Consequently the therapeutic index of this peptide is improved significantly respect to GS [26]. However, the two peptides with multiple Lys inversions have very weak hemolytic and also weak antimicrobial activities against Gram-negative and Gram-positive bacteria and fungi, resulting in low therapeutic indices respect to peptides with single Lys inversions in the GS14 ring (see Table 1).

Table 1. Biological activity against Gram-negative, Gram-positive bacteria and fungi, hemolytic activity, hydrophobicity (shown as Retention time measured with RP-HPLC) and LPS binding affinity of cyclic cationic peptide analogs of GS.

Peptide	Resid. No.	Linear sequence[c]	Ret. time (min)	Hemolytic	Gram-positive	Gram-negative	Fungi	LPS binding
GS	10	VOLFPVOLFP	34.1[a]	+++	++++	+++	+++	+
GS10	10	VKLYPVKLYP	30.8[a]	+++	++++	+++	+++	+
GS12	12	VKLKYPKVKLYP	23.4[a]	+	+	++	+	++
GS14	14	VKLKVYPLKVKLYP	31.4[a]	++++	+	+	+	++++
GS14*d*K_2	14	VKLKVYPLKVKLYP	39.8[b]	+++	++++	+++	+++	++
GS14*d*K_4	14	VKLKVYPLKVKLYP	37.8[b]	+	++++	+++	+++	++
GS14*d*K_9	14	VKLKVYPLKVKLYP	38.8[b]	++	++++	+++	+++	++
GS14*d*K_{11}	14	VKLKVYPLKVKLYP	37.1[b]	+	++++	+++	+++	++
GS14*d*K_2K_4	14	VKLKVYPLKVKLYP	33.1[b]	+	+	++	+	+
GS14*d*$K_2K_4K_9K_{11}$	14	VKLKVYPLKVKLYP	28.5[b]	+	+	+	+	+

According to Ref. [24[a], 25[a], 26[b]], °O stands for ornithine and underlined amino acids are D-enantiomers. Average activities as + (weak), ++ (medium), +++ (strong) and ++++ (very strong).

As it is obvious that membrane lipid bilayer is the primary target of GS and analogs, the examination of the various interactions between the peptides and phospholipid vesicles are crucial for understanding the mechanism of biological activity. Thus we have investigated the effect of the three biologically active ring-size analogs of GS (GS10, GS12 and GS14) on the thermotropic phase behavior of various phospholipid vesicles and on bilayer organization in gel and liquid-crystalline phases by DSC. Their ability to disrupt lipid membranes and permeabilize large unilamellar vesicles (LUVs) composed of PC [29] and PE/PG mixture [30, 31] were also determined. We were also curious to see whether or not the insertion of GS14, which has an increased size respect to GS, is more disruptive of bilayer cohesion. Finally, we investigated the relative potencies of GS10, GS12 and GS14 to inhibit the growth of the cell wall-less mollicute *Acholeplasma laidlawii* B (*A. laidlawii*). This microorganism belongs to the subbacterial family *Mycoplasmataceae*. It has no cell wall or intracellular membrane structure, and is surrounded by a single plasma membrane. Moreover, it is possible to alter its membrane lipid and the fatty acid composition with appropriate growth and media conditions. Membranes of *A. laidlawii* can be isolated by osmotic lysis [32]. The membrane lipid composition, its physico-chemical properties, the organization and dynamics of the lipid bilayer of this organism is the most extensively studied than any other membrane [33]. Previous work with GS have shown that the lipopolysaccharide (LPS)-containing cell wall or outer membrane of Gram-negative bacteria and the lipopeptidoglycan outer layer of Gram-positive bacteria may compete for the binding of peptides or even physically exclude them from the periplasmic space [12]. The absence of a cell wall or outer membrane allows peptides to interact directly with their primary target, the lipid bilayer of the limiting membrane. It would also enable some judgement on the possible influence of cell walls of conventional bacteria, aside from lipid composition related considerations [34]. Thus the use of *A. laidlawii* for assessing the antimicrobial potency of these peptides should obviate certain ambiguities and facilitate a molecular interpretation of the obtained results.

The relationships between the structural and physical properties of GS analog peptides, their relative antimicrobial and hemolytic activities and the mechanistic basis of the propensity to discriminate between bacterial and animal cell membranes are not fully understood. To address this issue, we have examined the structure-activity relationships of selected analogs, which were obtained by enantiomeric inversions of one or more of the Lys residues of GS14. We studied their conformational properties, their ability to destabilize and permeabilize model lipid membranes and their ability to inhibit the growth of *A. laidlawii*. Special emphasis has been addressed to understand the enhanced specificity of GS14*d*K_4 and to find possible correlation between its preference for bacterial membranes and its physicochemical properties.

Thus we utilized FTIR spectroscopy to characterize the interactions of GS14*d*K_4 with various single- and multi-component model membranes in order to obtain a better understanding of its interactions with membrane lipids and of its ability to discriminate between membranes of different cell types.

1.4. Aims

Experiments were carried out to examine:

(i) the effect of GS on the lipid phase transition behavior of dimyristoylphosphatidylcholine (DMPC) and on rotational dynamics of lipid acyl chain segments by spin label ESR spectroscopy as the sensitivity of ESR is suitable to study lipid chain dynamics and it has long been used to study protein-lipid interactions [35].

(ii) the influence of increasing peptide ring size on the propensity to perturb lipid phase behavior and whether a larger ring size and/or alterations in peptide conformation affect the ability of the peptide molecules to cause membrane disruption or membrane permeabilization or even bacterial cell lysis.

(iii) structure-activity relationships in a series of GS14 analogs obtained by single or multiple enantiomeric inversions of the Lys residues. We wanted to clear how the various peptide conformational properties affect the capacity of the analogs to destabilize and permeabilize model membranes and how they contribute to their biological activity.

(iv) the interaction of GS14*d*K_4 with single- and multicomponent lipid membranes to understand the molecular basis of its preferential lysis of bacterial rather than mammalian cells.

This thesis work is part of a broadly based long-term investigation of the feasibility of using the GS structural motif as a platform for the rational design of therapeutically useful antibiotic agents. We intended to broaden and refine our understanding of the physical principles underlying the membrane disruptive activity of these molecules. Particular attention has been given to define the factors that determine and influence their capacity to discriminate between bacterial and animal cell membranes. These principles will be used to guide future drug design decisions.

2. Materials and methods

2.1. Materials

Gramicidin S was purchased from Sigma (Munich, Germany). Ring-size analogs and diastereomeric Lys analogs of GS14 were synthesized by solid phase peptide synthesis using standard t-butyloxycarbonyl chemistry, purified by preparative RP-HPLC and converted to the HCl salt [26]. 14-SASL spin-label was kindly provided by Dr. Marsh (Max-Planck-Institut, Göttingen, Germany). Commercially available lipids were purchased from Fluka (Buchs, Switzerland) and Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Elaidate-homogenous *A. laidlawii* B membrane lipids were prepared by extracting the polar lipid fraction from cells cultured in avidin and elaidate-supplemented media [33]. Dielaidoyl monoglucosyldiacylglycerol (DE-MGDG) and dielaidoyl diglucosyldiacylglycerol (DE-DGDG) were isolated and purified using methods described by McMullen [36]. Other chemicals were highest purity reagent grade from BDH Inc. (Toronto, ON, Canada) and were used as received.

2.2. Electron spin resonance spectroscopy

We used spin label ESR spectroscopy to study the effect of GS on the chain melting transition of multilamellar DMPC vesicles and on rotational dynamics of chain segments close to the center of the bilayer, at various lipid/peptide ratios. 14-SASL, a freely diffusible single chain lipid analogue with the paramagnetic doxyl group on the C14 of its acyl chain was used to monitor the central region of the bilayer, at low label/lipid molar ratio (1:100). Experiments were carried out in close proximity and above the DMPC phase transtition ($T_m \cong 24°C$). DMPC (1 mg/mL) and 14-SASL were co-dissolved in chloroform/methanol (2:1) and the peptide (1 mg/mL) was dissolved in chloroform/methanol = 1:2. Then the peptide and the lipid + spin label solutions were mixed at desired ratios. After repeated vortexing, the sample was dried under N_2 stream and vacuum overnight. The dry film was suspended in 100 µL buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA, 1 mM Na-azide, pH 7.4) and vortexed for 30 s at 50 °C. The samples were filled into ESR sample capillaries (i.d. = 1 mm) and pelleted (3000 g) for 10 minutes at 4 °C. The supernatant was removed before the ESR measurements. This sample preparation is in principle identical with that of Prenner *et al.* [13] except for the spin labelling. ESR spectra were taken on a Bruker (Rheinstetten, Germany) ECS106 X-band spectrometer equipped with a TE102 cavity and a nitrogen gas flow temperature regulator. First harmonic spectra were recorded with 100 kHz field modulation, modulation amplitude of 0.1 mT, scan range of 10 mT

and microwave power of 10 mW, A/D resolution was 16-bit. Spectra were analyzed using software Igor (WaveMetrics, Lake Oswego, USA). The amplitudes and peak positions were determined by fitting a Gaussian distribution to the lineshapes around the extrema. The integrated intensities were calculated by numerical double integration after baseline correction.

2.3. Differential scanning calorimetry

Calorimetric measurements provide thermodynamic information on thermal events occurring in lipid or lipid-peptide samples while heating or cooling in a given temperature range. The phase transition endotherms can change in shape, cooperativity, total enthalpy, or shift to higher or lower temperatures upon the interaction of lipids with peptides. We measured the propensity of peptides to perturb the usual thermotropic phase behavior of the host lipid system and thereby evaluated the strength of interaction between peptide and lipid or lipid mixtures under investigation. The lipid/peptide samples were prepared by mixing the appropriate amounts of lipid dissolved in methanol/chloroform 1:2 and peptide dissolved in methanol, subsequently removing the solvent under a stream of N_2, and exposing the lipid/peptide films to high vacuum overnight. Hydrated MLVs were prepared by vortexing in buffer (10 mM Tris-HCl, 100 mM NaCl, 2mM EDTA, pH 7.4) at a temperature above the main phase transition of the lipid. Measurements were performed in a Nano-DSC Calorimeter (Calorimetry Sciences, Spanish Fork, UT) utilizing a scan rate of 10 °C/h. Sample runs were repeated at least three times. Data acquisition and analysis was done using Microcal DA-2 (MicroCal LLC, Northampton, MA) and Origin software (OriginLab Corporation, Northampton, MA). The peptide blanks dissolved in buffer exhibited no detectable thermal events over 0-90 °C, thus peptides do not undergo any cooperative thermal denaturation over this temperature range. The endothermic events observed in the lipid-peptide mixtures arise exclusively from phase transitions of the phospholipid.

2.4. Calcein leakage experiments

The different ability of each peptide to permeabilize bacterial or erythrocyte membranes is a very important factor in their biological activities as it is likely the manner by which antimicrobial peptides interact with the cells [37]. In our experiments we used 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1-palmitoyl-2-oleoylphosphatidylethanolamine/1-palmitoyl-2-oleoylphosphatidylglycerol (POPE/POPG) mixture vesicles (7:3 molar ratio). The latter mixture formed well-behaved defined LUVs of about the same size as POPC alone and was used to model *E. coli* membrane composition. Because of the high internal concentration, the fluorescence of the vesicle-entrapped dye calcein is self-quenched. When the peptides are

able to permeabilize vesicles calcein leaks out and due to dilution, increasing fluorescence is measured. Phospholipid vesicles were prepared by drying chloroform solutions of POPC or POPE/POPG under N_2 and overnight vacuum. Lipid films were hydrated with buffer used for the DSC experiments, but also containing a high concentration (70 mM) of calcein (Molecular Probes, Eugene, OR). The multilamellar vesicles were freeze-thawed several times and extruded through a 100-μm filter using a LipoFast apparatus (Avestin Inc., Ottawa, ON, Canada). The obtained LUVs were passed through a Sephadex G-50 column to remove calcein not trapped inside the vesicles. The peptide-induced leakage of the calcein was monitored by measuring the fluorescence intensity of calcein released into the buffer as a function of time by a Perkin-Elmer LS50B spectrophotometer (Beaconsfield, UK) at 25 °C, in quartz cells (i.d.=1 cm) and at slit widths of 3 nm for both excitation (496 nm) and emission (516 nm) [38].

2.5. *Acholeplasma laidlawii* B growth inhibition experiments

A. laidlawii B is a simple bacterium (Mollicute) without LPS-containing cell wall or peptidoglycan outer layers, which allows these peptides to interact directly with its cell membrane lipid bilayer, the presumed primary target of this class of antimicrobial peptides. The attack of the various peptides on the phospholipid bilayers of *A. laidlawii* inhibits bacterial growth to different extents. *A. laidlawii* cells were cultured in chloroform-extracted BSA-free media and cell growth was monitored turbidimetrically as previously described [33]. Various concentrations of the peptides were added to the culture medium just prior to the addition of a 10% (by volume) inoculum with cells in the mid log phase of growth. The effect of GS analogs on cell growth was monitored as a function of time. Cell growth in the presence of various concentrations of peptides is expressed as the maximum absorbance at 450 nm or as a percentage of the maximum growth relative to cells grown in the absence of peptide and ethanol.

2.6. Circular dichroism spectroscopy

The structural features of peptides can be estimated by monitoring the shape and the relative position of the minima of CD spectra. The spectrum is sensitive to changes in the β-sheet part of the peptide structure (215-225 nm) or to changes in the turn part (200-210 nm). Changes in the CD spectra correlate with changes in the backbone structure of cyclic peptides observed by NMR spectroscopy [26, 39]. According to previous studies, GS and GS14 exhibit CD spectra of double minimum pattern with large molar ellipticities at 206 and 223 nm. Comparison of the CD spectra of GS or GS14 with the spectra of new analogs gives information about their possible structure, changes in the peptide backbone or about relocation of the side

chains. CD spectra were recorded on a Jasco-J-720C spectropolarimeter (Tokyo, Japan) at 25 °C using quartz cells (i.d.=0.1 cm), using solutions containing 100 μM peptide and either buffer alone (of final concentrations of 10 mM Tris, 150 mM NaF and 0.1 mM EDTA, pH 7.5), equivolume mixtures of buffer and trifluoroethanol (TFE), or buffer containing 30 mM SDS.

2.7. Fourier transform infrared spectroscopy

Irradiation of the material by infrared light excites the vibrations of the chemical bonds, and absorption spectra are obtained. The dependence of the frequency of the peptide amide I infrared band on the polarity of its environment is used to determine the interaction of the peptides with the bilayer, the probable location and the depth of penetration of the peptide. As these peptides usually maintain their conformations in different solvents, frequency changes arise due to changes in the local environment of the peptide and not from alterations in the secondary or tertiary structure of the molecules. FTIR spectroscopy was performed on both dried films and liquid-based samples. The dried samples were thin films cast from methanolic solutions. For liquid-based peptide-lipid samples, the peptide and lipid were co-dissolved in methanol (lipid:peptide ratio=25:1). The solvent was removed in a stream of nitrogen and overnight evacuation. The mixture was hydrated with 75-100 μl of a D_2O buffer (50mM phosphate, 100mM NaCl, pH=7.4) and vortexed at temperatures well above the lipid phase transition. The dispersion was squeezed between CaF_2 windows of a heatable liquid cell. In the instrument, the sample temperature was regulated between -20° and 90°C by an external, computer-controlled water bath. Samples containing only the peptide (0.2-0.5 mg) were dissolved in the appropriate solvent and used as above. Spectra were recorded with Digilab FTS-40 infrared spectrometer (Digilab, Cambridge, MA) using acquisition parameters as described previously [40]. Spectra were analyzed with software from Digilab Inc. and OriginLab Corporation (Northampton, MA). In PS-containing samples, the headgroup carboxylate gives rise to a strong absorption band between 1610 and 1670 cm^{-1}, which can mask the peptide amide I absorption band [41]. Compensation for the interference was achieved by a weighted spectral subtraction procedure, using normalized spectra of the pure lipid samples. The procedure could be applied to spectra obtained at temperatures well above and well below the gel/fluid phase transition of the lipid-peptide mixture, because at those temperatures the peptide did not alter the shape of the PS ester C=O absorption bands. Residual FTIR samples were subsequently diluted with DSC buffer (lipid concentrations near 1.0 mg/ml), and were studied by high-sensitivity DSC (Calorimetry Sciences, Spanish Fork, UT) with 10°C/hr scans. Data obtained were analyzed with the Origin software (OriginLab Corporation, Northampton, MA).

3. Results

3.1. Perturbation of DMPC thermotropic phase behavior by GS

ESR spectra of GS-DMPC complexes (l/p = 5/1) labeled with 14-SASL were taken at temperatures below and above the lipid gel/liquid-crystalline phase transition to investigate the effect of GS on lipid phase transition (Figure 3). 14-SASL spin label monitors the central hydrophobic region of the bilayer predominantly in the fluid phase and produces isotropic 3-line ESR spectra when recorded at and above the chain-melting transition temperature of DMPC (T_m=24 °C=297 K). Spectra are consistent with fast isotropic rotation of 14-SASL in a fluid bilayer with no indication of membrane disruption caused by the peptide (spectra measured at 328, 313, 303, 298 K in Fig. 3 with solid lines). These spectra nevertheless indicate slower rotational motion of 14-SASL and also significant line broadening compared to those of pure lipid membranes (dotted lines). The spectrum pair on the top was recorded at a temperature (293 K), which corresponds to the gel phase of DMPC. The spectrum of the GS-DMPC complex (in Fig. 3 293 K solid line) is characteristic of a more fluid membrane respect to the pure spin-labeled DMPC bilayer at the same temperature (dotted lines). This fluidizing-rigidifying effect of GS is in agreement with the observation that it broadens the phase transition of DMPC [12].

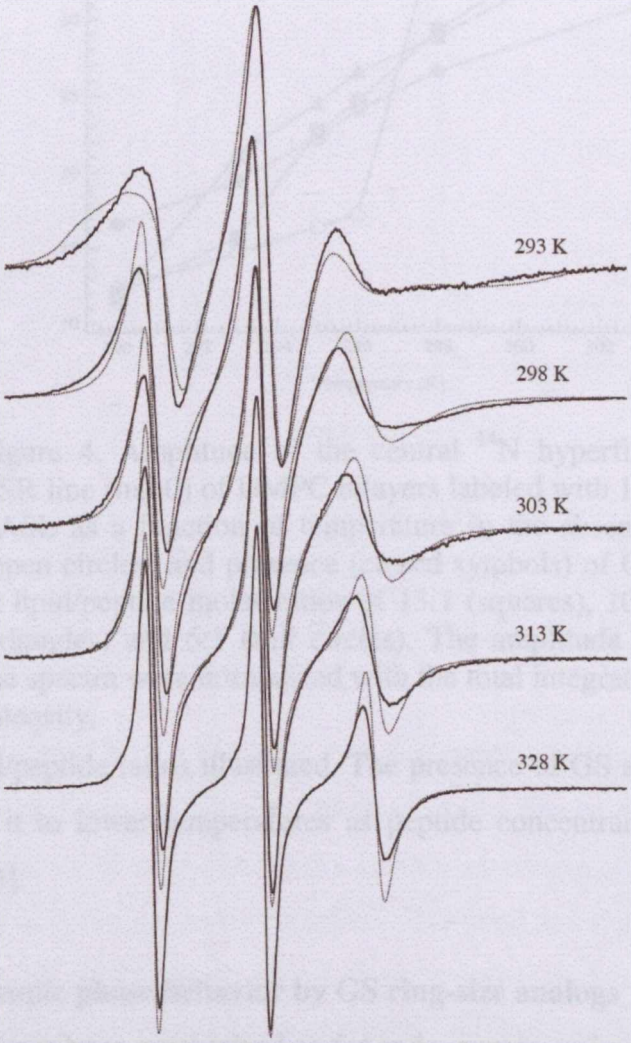

Figure 3. Conventional first derivative ESR spectra of GS/DMPC complexes labeled with 14-SASL. Spectra of DMPC MLVs were recorded in absence (dotted lines) and presence (solid lines) of GS at 5:1 lipid/peptide molar ratio. Spectra are scaled to the same maximum amplitude. Scan range shown is 8 mT.

Line broadening can be qualitatively measured from the amplitude of the central ^{14}N hyperfine ESR line. Broadening of the ESR lines of DMPC bilayers labeled with 14-SASL was measured as a function of temperature in the absence and presence of GS at various lipid/peptide molar ratios (see Fig.4). The amplitude of the ESR spectra was normalized with the total integrated intensity in order to compare different samples. Generally gel to fluid phase transition can be recognized as a steep change. The dependence of the normalized amplitude on temperature showed, that in agreement with Fig. 3, the hyperfine lines were in fact broader in presence than in the absence of the peptide at all lipid/peptide ratios illustrated. The presence of GS also broadened the phase transition and shifted it to lower temperatures as peptide concentration increased, in agreement with DSC studies [13].

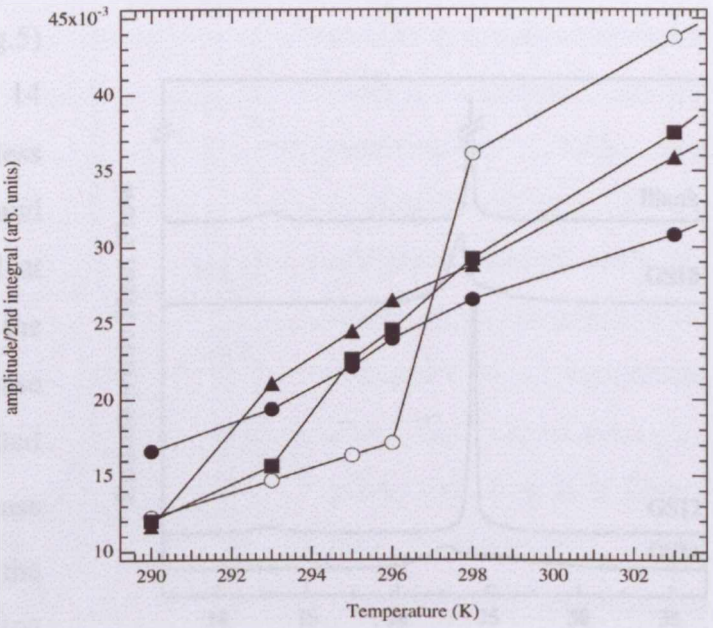

Figure 4. Amplitude of the central ^{14}N hyperfine ESR line (m_I=0) of DMPC bilayers labeled with 14-SASL as a function of temperature in the absence (open circles) and presence (closed symbols) of GS at lipid/peptide molar ratios of 13:1 (squares), 10:1 (triangles) and 6:1 (full circles). The amplitude of the spectra were normalized with the total integrated intensity.

3.2. Perturbation of phospholipid thermotropic phase behavior by GS ring-size analogs

A great number of GS analog peptides have been synthesized so far and we were curious if analogs with larger ring sizes with respect to GS had the same effect on the thermotropic phase behavior of different types of phospholipids as GS. We studied GS10, GS12 and GS14 in increasing concentrations (1-4 mol% i.e. 1:100-1:25 peptide:lipid molar ratio) and observed the magnitude of their characteristic effects on the thermotropic phase behavior of aqueous dispersions of two zwitterionic and an anionic phospholipid by DSC. DSC thermograms at the highest (1:25 peptide/ lipid molar ratio) peptide concentration are presented, as the characteristic differences in their effects were the most prominent under these circumstances.

Perturbation of DMPC thermotropic phase behavior by GS ring-size analogs

DMPC vesicles (see blank in Fig.5) exhibit two transitions: a pretransition at 14 °C, which is less enthalpic and less cooperative and a more cooperative and enthalpic main phase transition centered at ~24 °C. The pretransition corresponds to the conversion of a planar lamellar gel phase ($L_{\beta'}$) to the rippled gel phase with tilted hydrocarbon chains ($P_{\beta'}$). The main phase transition is caused by the conversion of the $P_{\beta'}$ phase to the lamellar liquid-crystalline (L_{α}) phase [42]. The temperature and enthalpy of the DSC endotherm observed for GS12/DMPC vesicles were essentially unchanged from that of DMPC blank and its cooperativity was only moderately reduced (Figure 5). In contrast, the incorporation of both GS10 and GS14 into the vesicles produced much broader, lower enthalpy two-component DSC endotherms, particularly in the case of GS14. Deconvolution of the DSC traces (data not shown) revealed a more cooperative sharp component at a lower temperature, and a less cooperative broad component centered at a higher temperature than the main phase transition of DMPC blank. According to prior studies of GS/DMPC mixtures [13], we interpret the sharp and broad components as the chain-melting phase transition of peptide-poor and peptide-rich phospholipid domains, respectively. Incorporation of GS10 and GS14 abolished the pretransition of DMPC whereas the incorporation of GS12 did not. These results suggest that GS12 perturbs the thermotropic phase behavior of DMPC bilayers to a much lesser extent than does GS10, and GS14 is more potent with respect to GS10.

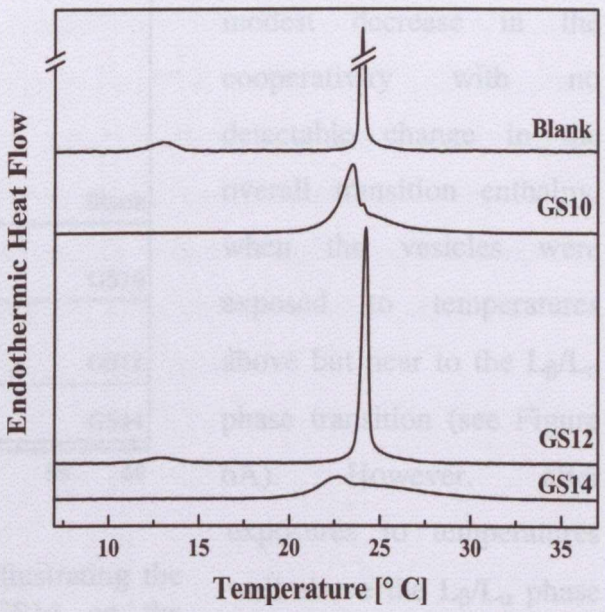

Figure 5. High-sensitivity DSC heating scans illustrating the effect of 4 mol% (1:25 peptide:lipid ratio) of GS10, GS12 and GS14 on the thermotropic phase behavior of DMPC MLVs.

Perturbation of DMPE thermotropic phase behavior by GS ring-size analogs

Aqueous dispersions of DMPE without extensive incubation at low temperatures exhibit a single fairly cooperative, energetic L_{β}/L_{α} phase transition centered near 50 °C [43]. The presence of these peptides had a very small effect on the phase transition of DMPE. They caused

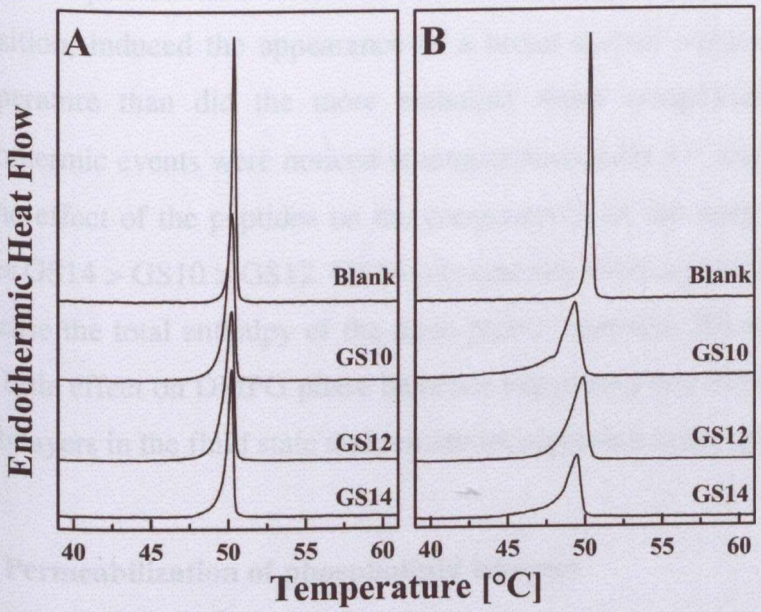

Figure 6. High-sensitivity DSC heating scans illustrating the effect of 4 mol% of GS10, GS12 and GS14 on the thermotropic phase behavior of DMPE MLVs. (A) DMPE MLVs not exposed to high temperatures (B) DMPE MLVs exposed to high temperatures (75 °C or higher)

only a slight reduction in the transition temperature and a modest decrease in the cooperativity with no detectable change in the overall transition enthalpy, when the vesicles were exposed to temperatures above but near to the L_β/L_α phase transition (see Figure 6A). However, after exposures to temperatures well above the L_β/L_α phase transition, the subsequent DSC heating scans revealed additional decrease in the temperature and cooperativity, and moderate change in the enthalpy (see Figure 6B). Subsequent recycling through the phase transition increased the effect of the peptides, suggesting that repeated exposure to high temperatures facilitates peptide incorporation into DMPE bilayers.

Perturbation of DMPG thermotropic phase behavior by GS ring-size analogs

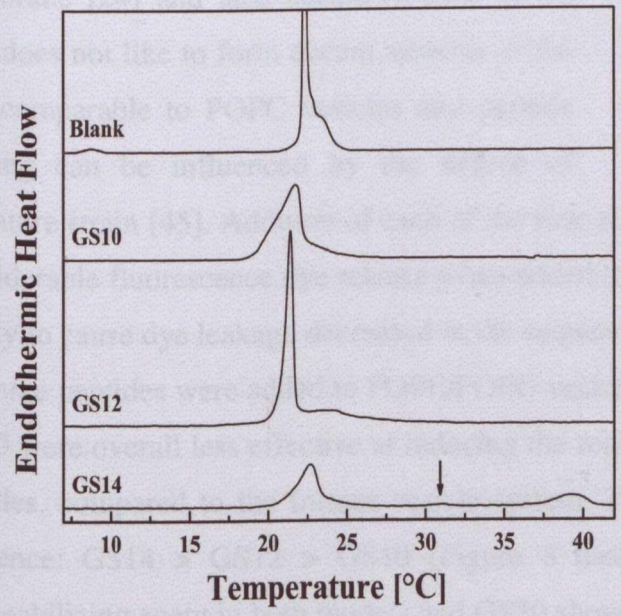

Figure 7. High-sensitivity DSC heating scans illustrating the effect of 4 mol % of GS10, GS12 and GS14 on the thermotropic phase behavior of DMPG MLVs.

Aqueous dispersions of DMPG, which have not been extensively annealed at low temperatures, exhibit two endothermic events upon heating, a less energetic pretransition near 14 °C and a more energetic main transition near 24 °C (Figure 7) [44]. The presence of each peptide produced

a two-component main transition endotherm. They all decreased the cooperativity of the main transition, induced the appearance of a broad second component, which occurred at a higher temperature than did the more enthalpic sharp component. In the case of GS14, further endothermic events were noticed at temperatures near 31° and 39 °C (Figure 7). The magnitude of the effect of the peptides on the cooperativity of the main phase transition decreased in the order GS14 > GS10 > GS12. GS14 substantially decreased whereas GS12 and GS10 appeared to increase the total enthalpy of the main phase transition. Recycling through the phase transition had little effect on DMPG phase behavior suggesting that these peptides readily incorporate into PG bilayers in the fluid state and remain incorporated in the gel state.

3.3. Permeabilization of phospholipid bilayers

GS ring-size analogs

To determine the relative ability of the three ring-size analogs of GS to permeabilize phospholipid bilayers, we measured the amount of entrapped calcein dye, released after the addition of 4 mol % peptide (1:25 peptide/lipid ratio) to LUVs composed of either POPC (simplified model for eukaryotic plasma membranes) or of a mixture of POPE/POPG (7:3 molar ratio). The latter mixture was used to model the composition of *E. coli* inner membrane [24] and also because POPE on its own does not like to form decent vesicles of the size comparable to POPC vesicles and peptide binding can be influenced by the degree of curvature strain [45]. Addition of each of the ring-size analogs in 4 mol % concentration caused considerable fluorescence dye release when added to POPC LUVs (Figure 8 white bars) and the ability to cause dye leakage decreased in the sequence: GS14 > GS10 > GS12. Same amounts of the three peptides were added to POPE/POPG vesicles. Interestingly, the analogs, in particularly GS10 were overall less effective at inducing the release of entrapped calcein from POPE/POPG vesicles, compared to the former vesicle system. Their relative effectiveness decreased in the sequence: GS14 > GS12 > GS10 (Figure 8 hatched bars). GS14 was the most effective permeabilizing agent in both models and GS10 showed the greatest phospholipid selectivity.

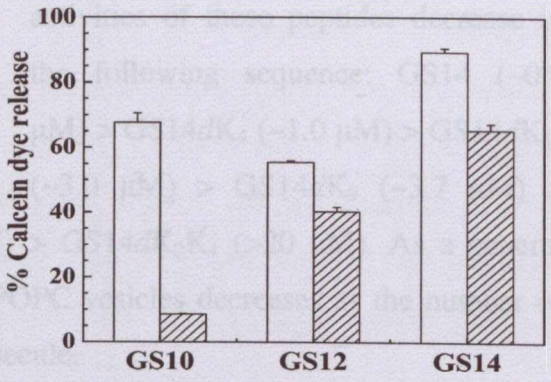

Figure 8. Percentage of entrapped calcein dye leakage from LUVs composed of either POPC (white bars) or POPE/POPG (7:3 molar ratio, hatched bars) upon the addition of 4 mol % GS10, GS12 or GS14.

Diastereomeric Lysine GS14 analogs

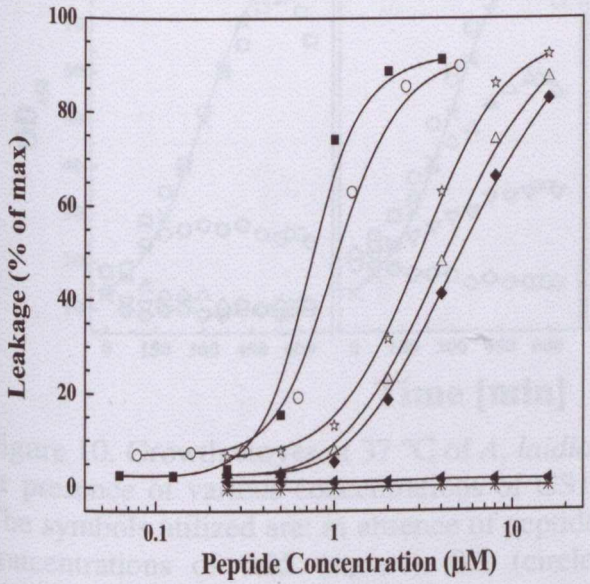

Figure 9. Peptide induced dye release from POPC LUVs. Data are presented as a function of peptide concentration for: GS14 (full square), GS14*d*K_2 (open triangle), GS14*d*K_4 (open circle), GS14*d*K_9 (full diamond), GS14*d*K_{11} (open star), GS14*d*K_2K_4 (cross), GS14*d*$K_2K_4K_9K_{11}$ (full triangle).

The permeabilization propensities of the Lys diastereomers of GS14 were also investigated by measuring their capacity to release calcein entrapped in POPC LUVs (see Figure 9). These experiments were performed at 37 °C, well above the T_m of POPC, to insure that we were determining lipid permeabilization in the biologically relevant lamellar liquid-crystalline state. The extent of peptide - induced permeabilization vary greatly and from the apparent LD_{50} values obtained, the membrane disruptive activities of these peptides decrease in the following sequence: GS14 (~0.9 μM) > GS14*d*K_4 (~1.0 μM) > GS14*d*K_{11} (~3.0 μM) > GS14*d*K_9 (~3.7 μM) > GS14*d*K_2 (~4.0 μM) > GS14*d*$K_2K_4K_9K_{11}$ (>10 μM) > GS14*d*K_2K_4 (>20 μM). As a general principle we can state that the permeabilization of POPC vesicles decreases as the number of Lys enantiomeric inversions increase in a peptide molecule.

3.4. Growth inhibition of *Acholeplasma laidlawii* B

GS ring-size analogs

We investigated the effect of the GS analogs on the growth of *A. laidlawii* B (in vivo testing system) to evaluate the relationship between the biophysical data of GS analogs and their antimicrobial properties. Utilizing this cell wall-free organism for such studies has major advantages as the antimicrobial peptides should have free physical access to the surface of the limiting membrane and extracellular structures should not compete with the lipid bilayer for peptide binding. The growth inhibitory potency of the three ring-size analogs was considerably different (see Figure 10). GS10 was fairly potent inhibitor of *A. laidlawii* growth, it suppressed growth slightly in the lowest peptide concentration tested (0.25 μM), strongly in 0.50 μM, and

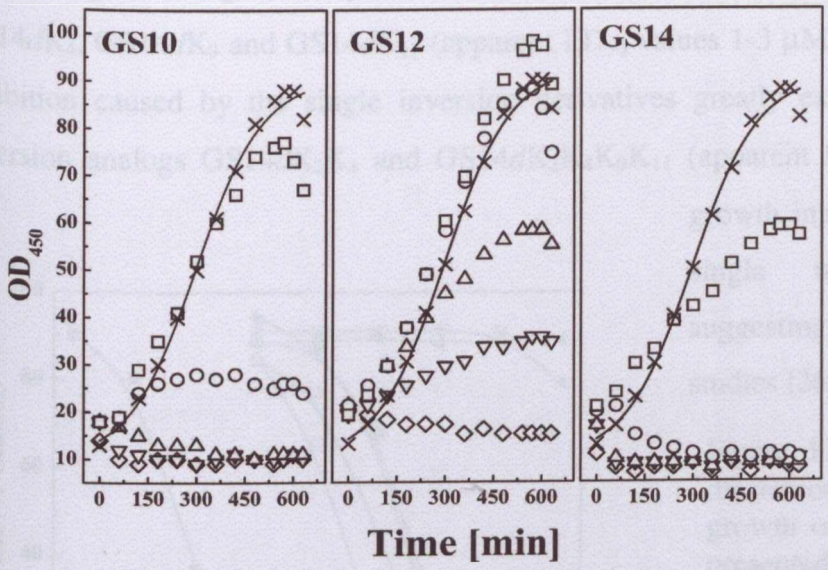

Figure 10. Growth curves at 37 °C of *A. laidlawii* B in the absence or presence of various concentrations of GS10, GS12 and GS14. The symbols utilized are: in absence of peptide (cross) and peptide concentrations of 0.25 (square), 0.5 (circle), 1.0 (up-pointing triangle), 2.0 (down-pointing triangle) and 4.0 μM (diamond) respectively in the growth medium.

completely inhibited growth when applied in concentrations of 1.0 μM and higher (Fig. 10). In contrast, GS12 had much weaker effect, with significant inhibition in 1.0–2.0 μM peptide concentrations and complete growth inhibition occurring only at 4.0 μM (Fig. 10). GS14 was a very potent inhibitor, because complete inhibition of growth occurred in all concentrations but the lowest (0.25 μM). Thus the relative sequence of growth inhibition potency resulted as: GS14 > GS10 > GS12. When sufficient peptide was added to the cultures to completely inhibit cell growth, the initial turbidity of the 10% (vol/vol) inoculum added to fresh culture media, fall to blank values. This suggests bacteriolytic rather than a static effect on *A. laidlawii* cells, indicating that these peptides presumably cause cell lysis.

Diastereomeric Lysine GS14 analogs

The effects of the Lys modified peptides on the growth of *A. laidlawii* B were also examined. Results indicate quite clearly that considerable variation in the growth inhibitory potency of these peptides exists. GS14 is a strong inhibitor of *A. laidlawii* growth at peptide concentrations near 0.2 μM with complete inhibition occurring at peptide concentrations near and above 0.5 μM. Whereas GS14*d*$K_2K_4K_9K_{11}$ expressed no discernable inhibition of *A. laidlawii* B growth at peptide concentrations up to 20 μM (Figure 11). Enantiomeric inversions of the Lys residues of GS14 markedly diminish the growth inhibitory potency and the magnitude of this effect seems to be generally correlated with the number and positions of the Lys enantiomeric inversions made. GS14 (no Lys inversions, its apparent LD_{50}~0.2 μM) inhibited *A.*

laidlawii growth significantly more than the single Lys inversion derivatives: GS14*d*K_2, GS14*d*K_4, GS14*d*K_9 and GS14*d*K_{11} (apparent LD_{50} values 1-3 μM) (see Fig. 11). However, the inhibition caused by the single inversion derivatives greatly exceeds those of the multiple inversion analogs GS14*d*K_2K_4 and GS14*d*$K_2K_4K_9K_{11}$ (apparent LD_{50} values > 20 μM). The growth inhibitory potencies of the four single inversion analogs differ, suggesting in accordance with other studies [26] that they are inequivalent.

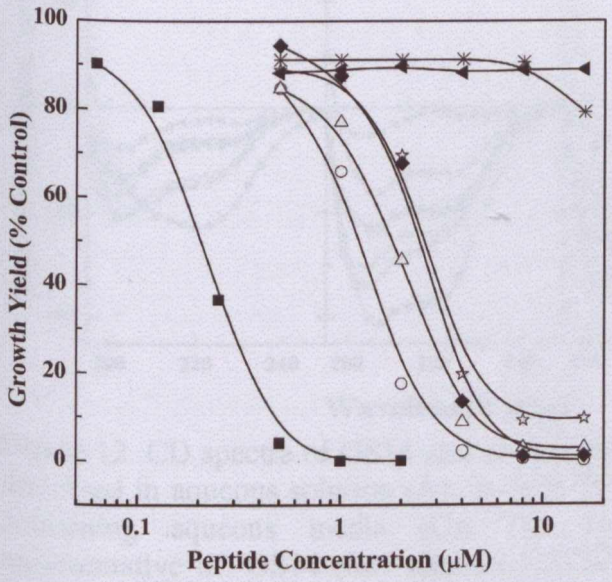

Figure 11. Effect of GS14 and its diastereomeric analogs on the growth of *A. laidlawii* B. Data are presented as a function of peptide concentration for: GS14 (full square), GS14*d*K_2 (open triangle), GS14*d*K_4 (open circle), GS14*d*K_9 (full diamond), GS14*d*K_{11} (open star), GS14*d*K_2K_4 (cross), GS14*d*$K_2K_4K_9K_{11}$ (full triangle).

3.5. Circular dichroism spectroscopic studies of diastereomeric Lysine GS14 analogs

CD spectra of GS14 in aqueous and membrane mimetic media exhibits large negative molar ellipticity values between 210 and 230 nm, which is not surprising as GS14, like GS, exists predominantly in an antiparallel β-sheet structure with type II' β-turns [28]. Molar ellipticities of GS14 are considerably greater than those exhibited by all other derivatives (see Fig. 12A), suggesting that enantiomeric inversion of any of the Lys residues causes significant distortion of the stable antiparallel β-sheet conformation. GS14 analogs with single Lys inversions all exhibit higher molar ellipticity values compared to GS14*d*K_2K_4, which in turn exhibits higher molar ellipticity values than those of GS14*d*$K_2K_4K_9K_{11}$ (Figure 12A). This indicates that conformational distortions of the GS14 structure caused by enantiomeric inversion of the Lys residues increases progressively with the number of such inversions. Moreover, molar ellipticity values of the single-substitution derivatives decrease in the order GS14dK_{11} ≥ GS14dK_4 > GS14dK_2 ≥ GS14dK_9, indicating that the distortions of the GS14 structure are inequivalent. Molar ellipticities of GS14*d*K_4 and GS14*d*K_{11} are generally comparable in magnitude as are those of GS14*d*K_2 and GS14*d*K_9. This suggests that the conformational distortions caused by enantiomeric inversions of the K_4 and K_{11} residues of GS14 are

comparable but different from those caused by enantiomeric inversions of the K_2 and K_9 residues.

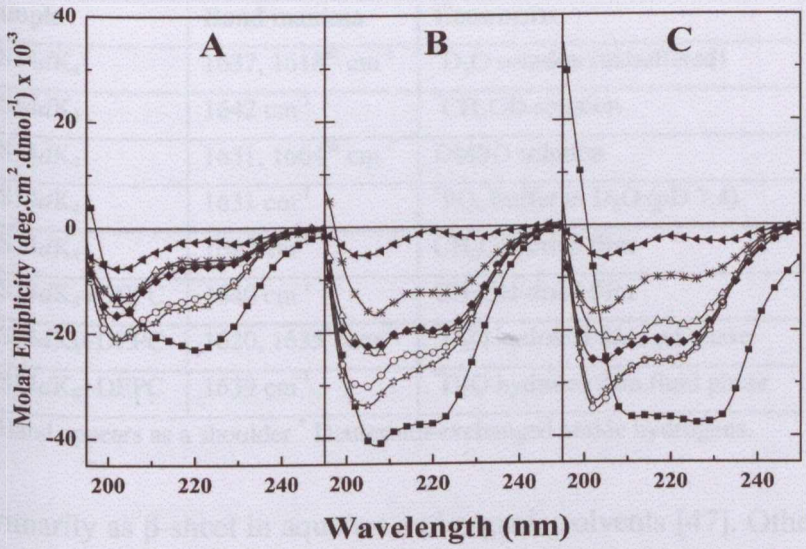

Figure 12. CD spectra of GS14 and its diastereomeric derivatives dissolved in aqueous solution (A), in 50% TFE (B) and in SDS-containing aqueous media (C). The spectra shown are representative of: GS14 (full square), GS14*d*K_2 (open triangle), GS14*d*K_4 (open circle), GS14*d*K_9 (full diamond), GS14*d*K_{11} (open star), GS14*d*K_2K_4 (cross), GS14*d*$K_2K_4K_9K_{11}$ (full triangle).

These peptides (except for GS14*d*$K_2K_4K_9K_{11}$) exhibited significantly higher molar ellipticity values when dissolved in 50 vol% TFE (Fig. 12B) - a solvent with structure-inducing properties [26] - and in SDS-containing aqueous media (Figure 12C) than when dissolved in aqueous buffer alone. Increases in molar elliplicity of peptides dissolved in TFE-containing solvents indicate that the peptides (except GS14*d*$K_2K_4K_9K_{11}$) adopt somewhat distorted conformations in aqueous media that are receptive to the structure-inducing properties of TFE. Molar ellipticities in SDS-containing aqueous media are comparable to those in 50 vol% TFE. Such behavior is commonly observed with water-soluble amphipathic peptides that have the capacity to partition between aqueous media and lipid bilayers. It reflects the capacity for inducible conformational changes in response to changes in the polarity of the local environment [46].

3.6. Infrared spectroscopic studies on the interactions of GS14*d*K_4 with single-component lipid bilayers

In order to characterize the interactions of GS14*d*K_4 -a particularly promising member of the diastereomeric Lys GS14 analogs- with membranes and to understand the molecular basis of its preference to lyse bacterial cells we have investigated the interactions of GS14*d*K_4 with lipid model membranes by FTIR spectroscopy. The amide I band frequencies of this peptide are strongly influenced by the local environment. Comparison of the spectra of GS14*d*K_4 in solvents of different polarity and hydrogen bonding capacity (D_2O, CH_3OD, DMSO) indicate an upward shift of the amide I band frequency as the polarity of the solvent decrease (Table 2). These

frequencies and frequency shifts are comparable to the solvent-induced changes observed in previous FTIR spectroscopic studies of GS [12, 18].

Table 2. Amide I band maxima of the Hydrochloride salt of GS14*d*K$_4$.

Sample	Band maxima	Comments
GS14*d*K$_4$	1637, 1618^{sh} cm^{-1}	*D_2O solution (unbuffered)
GS14*d*K$_4$	1642 cm^{-1}	*CH_3OD solution
GS14*d*K$_4$	1651, 1664^{sh} cm^{-1}	DMSO solution
GS14*d*K$_4$	1631 cm^{-1}	*PO_4 buffer in D_2O (pD 7.4)
GS14*d*K$_4$	1642 cm^{-1}	CH_3OH-dried film
GS14*d*K$_4$+DEPC	1640 cm^{-1}	CH_3OH-dried film
GS14*d*K$_4$+DEPC	1620, 1635^{sh} cm^{-1}	*D_2O hydrated film,gel phase
GS14*d*K$_4$+DEPC	1639 cm^{-1}	*D_2O hydrated film,fluid phase

^{sh} Band appears as a shoulder * Deuterium-exchanged amide hydrogens.

The dominant peak exhibited by the peptide dried from methanol solution at 1642 cm^{-1} (Table 2) was assigned to the amide I vibrations of hydrogen-bonded residues forming the antiparallel β-sheet portion of the GS14*d*K$_4$ molecule. It indicates that GS14*d*K$_4$ exists primarily as β-sheet in aqueous and organic solvents [47]. Other smaller spectral components in this region (1660-1680 cm^{-1}) arose probably from the amide I vibrations of *d*Pro and Tyr forming the type II´ β-turns [18] and were not the focus of this work. An unexpected low-frequency component at 1618 cm^{-1} appeared on the spectra of GS14*d*K$_4$ in D_2O solution and in PC bilayers in gel phase. However, in fluid phase this band disappeared, suggesting that the low-frequency component probably arises from a conformationally distinct peptide population, which is stabilized by gel phase lipids (see Table 2). This band is not due to the aggregation of peptide β-sheets, because in those cases a *pair* of sharp amide I bands at 1618 and at 1680 cm^{-1} emerge, which are together characteristic for aggregated peptide β-sheet [24, 45]. The low-frequency component is of interest, because the changes in amide I band of GS14*d*K$_4$ in different bilayers were the combined result of the migration of peptide molecules from aqueous solution into the polar/apolar interfacial regions of the bilayer and the decline of the conformationally distinct peptide population induced by the fluid lipid phase.

Interactions of GS14dK$_4$ with zwitterionic bilayers

At low temperature a sharp major component centered 1620 cm^{-1} was the dominant feature of amide I band of a DMPC/GS14*d*K$_4$ (25:1) mixture (Figure 13). This low-frequency component persisted until the onset of L_β/L_α phase transition, but its intensity decreased progressively as the phase transition proceeded, when it was completed, the feature disappeared and the amide I band envelope consisted of a broad peak centered near 1637-1640 cm^{-1} (see Figure 13). The upward shift of the amide I frequency of gel phase to fluid phase indicate a change in the polarity of the environment around the peptide. The disappearance of the low-

frequency component at higher temperatures suggests also, that the peptide partitioned into the PC bilayer interface.

The interactions of GS14*d*K_4 with single-component zwitterionic DMPE model membranes were also examined (see Figure 14). Below the lipid L_β/L_α phase transition the mixture exhibited amide I bands containing a sharp component at 1620 cm^{-1}, similar to that observed with PC-GS14*d*K_4 mixtures. The low-frequency band persisted until the onset of the phase transition but decreased markedly in intensity upon further heating. Concomitantly a broader amide I component near 1637 cm^{-1} appeared. Unlike the PC-GS14*d*K_4 mixtures, the sharp low-frequency amide I band persisted above the phase transition, which suggests that a sizeable fraction of the peptide did not penetrate and/or partition into liquid-crystalline PE bilayers.

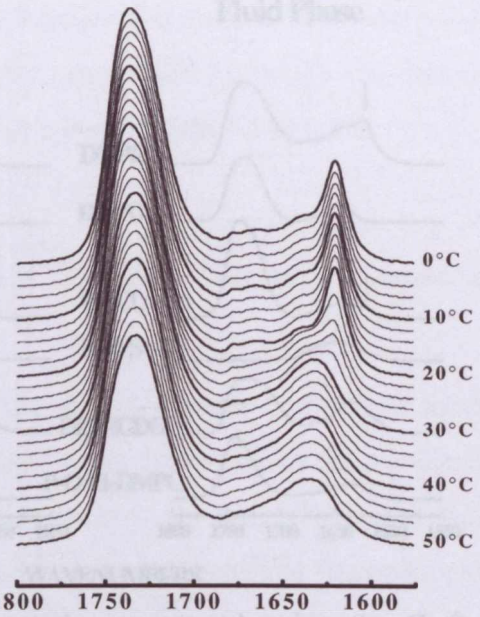

Figure 13. Interaction of GS14*d*K_4 with DMPC model membranes. Stacked plot illustrating the temperature-dependent changes in the C=O stretching and amide I regions of FTIR spectra exhibited by aqueous dispersions of GS14*d*K_4 /DMPC mixtures (1:25).

Interactions of GS14dK$_4$ with anionic bilayers

In the gel phase, the amide I band of DMPG-based lipid-peptide mixtures was centered near 1638 cm^{-1} and coincident with the melting of the lipid, its frequency drifted upward to values near 1644-1646 cm^{-1} (Figure 14). The gel phase frequency of the mixture is higher than the frequency of the peptide in aqueous solution, indicating interactions between GS14*d*K_4 and gel phase PG bilayers. The sharp low-frequency amide I component was absent in the gel phase spectrum. The fluid phase frequency of GS14*d*K_4-PG mixture (1644 cm^{-1}) was higher than that observed when GS14*d*K_4 interacted with the fluid PC or PE bilayers, which suggests stronger interactions between the peptide and the L_α phase of anionic lipids. The results suggest, that the membrane interactions, which stabilize the conformationally distinct GS14*d*K_4 population do not occur when the peptide is associated with PG bilayers.

Interestingly, mixtures of GS14*d*K_4 with the anionic lipids TMCL and DMPS both exhibited a sharp, low-frequency amide I band near 1620 cm^{-1}, comparable to PC- and PE-based mixtures (see Figure 14). Upon heating to temperatures well above their respective gel/fluid

phase transitions, the intensity of the low-frequency component diminished with a concomitant emergence of a broader amide I band centered near 1644 cm^{-1}. In TMCL-based mixtures, the sharp component persisted until 10 °C above the lipid phase transition, although its intensity declined (Figure 14). The behavior of GS14*d*K_4/TMCL and GS14*d*K_4 /DMPS mixtures, according to these observations, were more comparable to that of GS14*d*K_4/PC bilayers, however, the amide I frequencies in fluid phase of the TMCL- and PS-based mixtures were 4-6 cm^{-1} higher than those of PC-mixtures, indicating greater partitioning and/or deeper penetration into TMCL and PS bilayers in fluid state.

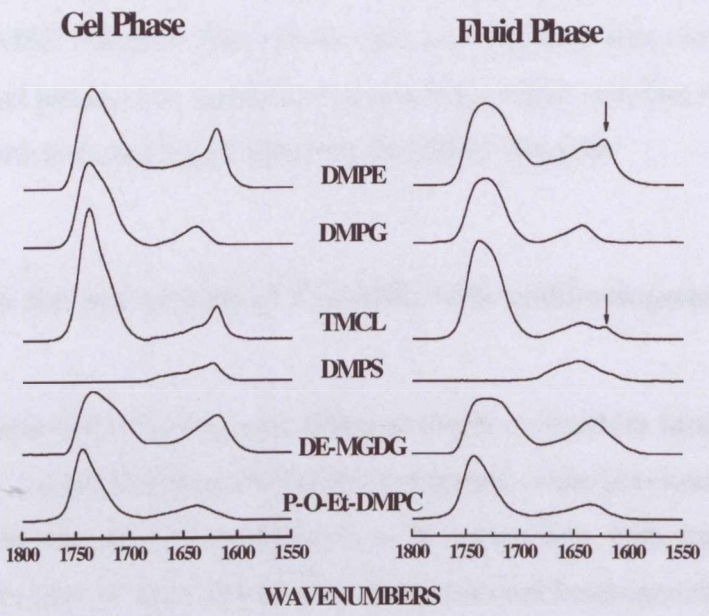

Figure 14. Representative spectra showing the C=O stretching and amide I regions of FTIR spectra exhibited by 1:25 mixture of GS14*d*K_4 and the indicated phospholipid or glycolipid bilayers. The data are typical of those obtained at temperatures 10 °C below (gel phase) and 5 °C above (fluid phase) the onset or completion of the lipid gel-liquid-crystalline phase transitions of the various mixtures.

Interactions of GS14dK$_4$ with nonionic and cationic bilayers

Below the L_β/L_α phase transition, amide I bands of mixtures composed of GS14*d*K_4 and nonionic glycolipids DE-MGDG (Figure 14) or DE-DGDG (spectra not shown) consisted of a skewed band envelope with a peak at 1620 cm^{-1}. In fluid phase, the amide I band adopted a more symmetrical shape at 1634 cm^{-1}. The spectroscopic changes were the result of a fluid phase-induced decline of the component at 1620 cm^{-1} and a concomitant increase in the intensity of a broader component at 1634 cm^{-1}. The changes were similar to those of GS14*d*K_4 with PC and with anionic TMCL and PS bilayers. However, the frequencies of the peptide in fluid nonionic bilayers were lower, than those with PC bilayers, and markedly lower respect to anionic lipids. This suggests that the interaction of GS14*d*K_4 with the L_α phase of nonionic bilayers was probably weaker than with any of the zwitterionic and anionic phospholipid bilayers.

The mixture of GS14*d*K_4 with cationic lipid P-O-Et-DMPC exhibited a broad amide I band centered near 1633-1635 cm^{-1}, and this frequency was essentially insensitive to changes in

the temperature and phase state of the host lipid bilayers (Figure 14), indicating that GS14dK_4 may not be interacting with P-O-Et-DMPC bilayers. The mixture did not exhibit a sharp low-frequency component at 1620 cm^{-1} in gel phase, thus membrane interactions which stabilize the conformationally distinct peptide population do not occur with P-O-Et-DMPC bilayers.

3.7. Infrared spectroscopic studies on the interactions of GS14dK_4 with multi-component lipid bilayers

After studying the interactions between GS14dK_4 and different single-component model bilayers, its interactions with a number of multi-component bilayers composed of the previously studied phospholipids were investigated, in order to examine how its interaction with lipid bilayers are likely to be affected by the types of lipid head group compositional heterogeneity, which characterizes natural cell membranes.

Interactions of GS14dK$_4$ with A. laidlawii B membrane lipids

A. laidlawii membrane polar lipid extract is composed mainly of nonionic glycolipids MGDG and DGDG (60-70 mol%), of anionic PG (20-30 mol%) and small amounts of anionic phosphorylated glycolipids and amino-acylated PGs [48]. At low temperatures, the *A. laidlawii* lipid extract with GS14dK_4 exhibited an amide I band envelope which contained a small sharp component centered at 1620 cm^{-1} and a broader component centered near 1636 cm^{-1} (Figure 15A). Upon heating, both components diminished in intensity were replaced by a single broader component centered near 1642 cm^{-1}. The latter frequency was lower, than the one observed for GS14dK_4/PG bilayers, but much higher than for nonionic glycolipids (see Figure 14). This indicates that the interaction of the peptide with fluid phases of *A. laidlawii* bilayers is stronger than with the pure glycolipid components but weaker than with PG. This result provides evidence that PG facilitates stronger interactions between GS14dK_4 and the lipid membrane.

Interactions of GS14dK$_4$ with Gram-negative Escherichia coli mimetic bilayers

PG-facilitated membrane interaction was also apparent when the peptide interacted with PE:PG (7:3) lipid mixture which approximates the composition of *E. coli* cell membrane. GS14dK_4 exhibited a broad amide I band centered near 1629 cm^{-1} in gel phase and a narrower band envelope near 1638 cm^{-1} in fluid PE:PG membranes (Figure 15B). Further analyses indicated that the broad amide I band seen at low temperatures was a summation of two

components of comparable intensities at 1620 and 1634 cm^{-1}. While lipids melted the 1620 cm^{-1} component progressively diminished in intensity and was replaced by a component at 1642 cm^{-1}, which coexisted with the component at 1634 cm^{-1}. These spectroscopic results differ markedly from those observed when GS14dK$_4$ interacted with PE or PG alone (Figure 14).

Interactions of GS14dK$_4$ with model bilayers of yeast and other fungi

We examined the interactions of GS14*d*K$_4$ with model membranes composed of equimolar amounts of PC, PE and PS, mimicking the membranes of many yeast and other fungi (Figure 15C). At temperatures well below the phase transition the sharp amide I component appeared near 1620 cm^{-1}, similar to what was observed when the peptide interacted with each of the pure lipid components alone. However, in fluid phase the amide I band was centered near 1640-1642 cm^{-1}, frequencies below those of the peptide with liquid-crystalline PS bilayers, but above those observed with either fluid PC or PE bilayers (Figures 13A and 14). Evidently PS, like PG, is capable of facilitating GS14*d*K$_4$/membrane interactions.

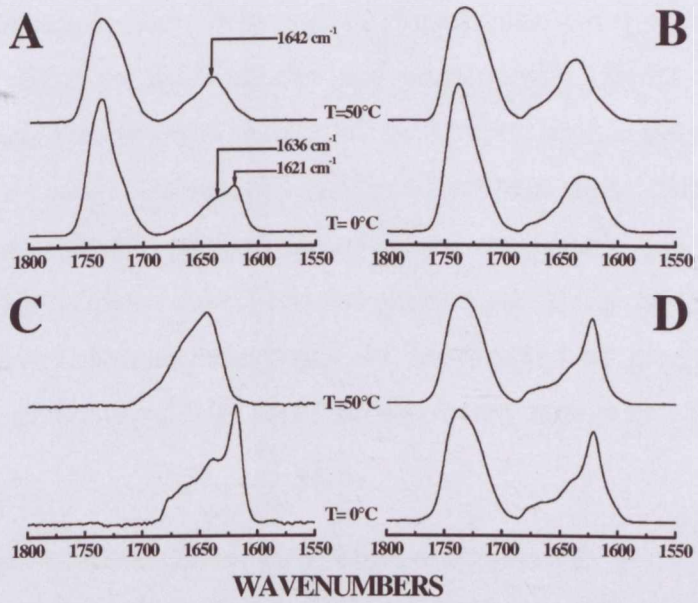

Figure 15. C=O stretching and amide I regions of FTIR spectra exhibited by GS14*d*K$_4$ with multi-component lipid bilayers. The absorbance spectra acquired in gel (low temperature) and fluid (high temperature) phases of the various mixtures are shown. Data are presented for mixtures of GS14*d*K$_4$ with (A) *A. laidlawii* B membrane lipids, (B) DEPE and DEPG (7:3), (C) DEPC, DEPE and DEPS (1:1:1) and (D) DMPC membranes containing ~30% cholesterol.

Interactions of GS14dK$_4$ with bilayers mimicking mammalian membranes

The effect of GS14*d*K$_4$ on cholesterol-containing PC membranes was examined to estimate its probable interactions with outer monolayers of mammalian cell membranes. Mixtures of GS14*d*K$_4$ with DMPC membranes containing ~30 mol% cholesterol exhibited amide I band envelopes that were dominated by the sharp low-frequency amide-I component at 1620 cm^{-1}, (see Figure 15D) a feature proved to be characteristic to most gel phase lipid

bilayers. This amide I feature persisted to temperatures well above the typical broad phase transition of cholesterol-containing PC membranes, with only a small decline in its relative intensity over the entire temperature range studied (–2°C and 75°C). These results differed markedly from those observed when GS14*d*K_4 interacted with membranes composed of PC alone (see Figure 13), and suggest that the presence of cholesterol drastically inhibits GS14*d*K_4 interaction with liquid-crystalline PC bilayers. The diminished propensity of this peptide to interact with cholesterol-containing membranes is probably the main reason why it exhibits significantly lower hemolytic activity than do peptides such as GS, GS10 and GS14.

4. Discussion

The mode of interaction of peptide antibiotics with biological membranes is an integral part of their mechanism of biological activity. To fully understand the nature and the strength of the interactions between a peptide and the lipid bilayer a detailed examination of the peptide structure and its position within the bilayer is required. Moreover, it is necessary to investigate the effect of these membrane-associated peptides on the surrounding lipid molecules. How effectively can peptides change the thermotropic phase behavior, the organization and dynamics of the bilayer lipids and induce permeation of lipid vesicles and consequently disrupt the integrity of various biological membranes? The results of such studies contribute significantly to our understanding of the molecular basis of the biological activities of cationic antimicrobial peptides. Furthermore, the obtained data elucidate several desirable and undesirable peptide properties and many of the factors, which influence and determine peptide selectivity. In turn, such information can guide drug design decisions by broadening the knowledge base required for the development of a new generation of therapeutically useful peptide-based antibiotics.

Disruption of membrane integrity and permeation of model and biological membranes

Numerous studies have proposed that disruption of the barrier properties of cell membranes is the basis of the antimicrobial and hemolytic properties of membrane-targeted antimicrobial peptides such as GS [9, 10] It is generally assumed that these molecules partition into the periplasmic membranes of prokaryotic and eukaryotic cells, where they degrade membrane barrier function by disrupting membrane structure and integrity.

One of the more notable manifestations of the disruption of membrane integrity, arising from the association of the peptide with the bilayer, is the change in the usual gel to fluid phase transition parameters (i.e. T_m, $\Delta T_{1/2}$, and ΔH_{cal}) of the membrane lipid constituents. Thus, a combination of DSC and ESR spectroscopy were used to investigate the effect of GS on the properties of gel/liquid-crystalline phase transitions of various phospholipids, in order to estimate and rank relative strengths of the interaction of GS with different types of lipids. Our studies clearly show that, the incorporation of GS into DMPC model membranes resulted in a concentration-dependent broadening of the lipid gel/liquid-crystalline phase transition and a shifting of its mid point to lower temperatures (Figures 3 and 4). These observations were consistent with results of the calorimetric and FTIR spectroscopic studies reported by Prenner *et al.* and by others [13, 14, 37]. This behavior is undoubtedly a reflection of the perturbation of

lipid hydrocarbon chain packing caused by the insertion of the peptide into the membrane structure [13]. However, other studies have shown that the effect of GS on lipid thermotropic phase behavior is also strongly dependent on the phase state, structure and charge of the lipid polar headgroup [12, 13]. The overall consensus of such work is that, GS interacts more strongly with and/or penetrates more deeply into anionic phospholipids such as PG than into zwitterionic phospholipids such as PC and PE. Furthermore, GS interacts more strongly with liquid-crystalline than with gel state lipid bilayers and more strongly with disordered membranes such as PC than with PE membranes which, nominally, form more ordered bilayer structures [12, 13]. The results of our studies of the interaction of the ring-size analogs GS10, GS12 and GS14 with DMPC, DMPE and DMPG bilayers (see section 3.2. of this thesis) are also consistent with the consensus arrived at in comparable studies of GS interactions with lipid bilayers. However, although the overall lipid specificity of the effects of the three peptides (i.e. DMPE < DMPC < DMPG) was broadly similar to that of GS, the three peptides differed quantitatively as regards their interactions with the above examined phospholipids (GS14 > GS10 > GS12). The relatively weak effects of GS12 on the various model lipid membranes is consistent with its relatively weak antibiotic and hemolytic activity [22], and is probably a reflection of the combined effect of its lower amphipathicity and β-sheet content (see below).

One of the general features of these AMPs is their capacity to permeabilize cells and phospholipid vesicles. Our studies of peptide-induced permeation of model membranes were intended to evaluate and rank the relative potencies of the various peptides to permeabilize lipid membranes. The peptide-induced permeation of zwitterionic PC vesicles (the major phospholipid component of the outer leaflet of eukaryotic plasma membranes) and anionic POPE/POPG vesicles (the major lipid components of bacterial cell membranes) indicated that the potency of the three ring-size analogs to permeabilize phospholipids vesicles decreses as: GS14 > GS10 > GS12. This is the same order as their capacity to inhibit the growth of the cell wall-less mollicute *Acholeplasma laidlawii* B, their hemolytic activities [22], and their capacity to modulate lipid thermotropic phase behavior (see above). These results suggest that the capacity of these peptides to permeabilize the phospholipid model membranes as well as the membranes of human erythrocytes and *A. laidawii* B cells are strongly correlated with their capacity to perturb the phase behavior and organization of lipid bilayers.

Interestingly, however, the results of our permeabilization studies do not correlate nicely with the previously published work which indicated that the antibiotic activity of the tetradecameric ring size analog GS14 against various Gram negative, and positive bacteria is very weak [22]. The poor antibiotic activity of GS14 reported by Kondejewski *et al.* [22] also

contrasts sharply with the results of our studies of the growth inhibitory potency of GS14 against *A. laidlawii*. However, in examining the possible basis of the apparent discrepancy between the two sets of experimental observations, two important factors need to be considered. First, unlike the cell membranes of Gram negative and Gram positive bacteria, the outer surfaces of erythrocytes, *A. laidlawii* B and the phospholipid vesicles are not covered by lipopolysaccharides (Gram negative bacteria) or peptidoglycan moieties (Gram positive bacteria). Thus, it is possible that the weak antibiotic activity of GS14 as determined by Kondejewski *et al.* may actually be reflecting preferential GS14 interaction with lipopolysaccharide and peptidoglycan moieties on the outer surfaces of these bacterial cells instead of their periplasmic membranes. Such a possibility is supported by the results of previous work indicating that GS14 has a very high propensity for binding to LPS [26]. Second, recent studies by Lee *et al.* [49] indicate that GS14 has a very high innate propensity for self-association in aqueous media, a result consistent with previous suggestions of its relatively low monomeric solubility in aqueous media [24]. It is therefore possible that the low antibiotic activity of GS14 against the Gram negative and positive bacteria may actually be reflecting the combined effect of its interaction with lipopolysaccharides and/or peptidoglycans and solubility limitations under the particular conditions of the assay. This particular issue is currently outstanding and needs to be addressed further.

Membrane permeation studies were also performed with a series of Lysine diastereomeric analogs of the tetradecameric GS14 (GS14, GS14*d*K_4, GS14*d*K_{11}, GS14*d*K_9, GS14*d*K_2, GS14*d*K_2K_4 and GS14*d*$K_2K_4K_9K_{11}$) against the POPC vesicle system. This series of peptides had essentially identical amino acid compositions and sequences, but their amphipathic properties were altered by L- to D-substitutions of one or more of their Lys residues. This series of compounds suited thus well for an examination of how peptide amphipathicity influences the capacity to permeabilize lipid membranes. Our studies indicate that the degree of POPC permeabilization varied significantly among the seven peptides and their membrane disruptive activities decreased markedly with the number of enantiomeric substitutions. These results correlated strongly with the effects of the anomeric inversions on lipid bilayer perturbation propensities, with the capacity to inhibit the growth of *A. laidlawii* B, and with the hemolytic activities of these peptides. Indeed, the results of these experiments and the accompanying studies on their interactions with lipids were generally consistent with the predicted effects of the enantiomeric inversions on the physicochemical properties of these peptides (see below).

Factors and characteristics that affect the biological activity of antimicrobial peptides derived from the GS structural motif

The basic elements of GS structural motif are a pair of antiparallel aligned, alternating sequences (designated as "side-sequences") of hydrophobic and charged amino acid residues connected head-to tail by a pair of type II' β-turns formed by the sequence X-P, where X can be any D-amino acid except proline. For this structural motif, it was predicted [50] and experimentally verified [27], that when the side-sequences contain an odd number of amino acid residues, stereochemical factors will be conducive to folding into fairly stable conformations in which the side-sequences form anti-parallel aligned β-sheets which may be stabilized by cross-ring hydrogen bonds. These considerations constitute the molecular basis of the high conformational stability of GS, which has side-sequences composed of three amino acid residues (Val-Orn-Leu). Based on the stereochemical considerations described above, one can make a number of predictions about the structural properties of GS derivatives that are relevant to the work presented in this thesis. First, GS derivatives with side-sequences composed of an odd-number of amino acid residues should fold into conformations of greater stability and higher β-sheet content than those with side-sequences composed of an even number of amino acid residues. Ring size derivatives of the GS-structural motif should thus exhibit a distinct periodicity in their conformation, conformational stability, and β-sheet content, based on whether their side-sequences contain an odd- or even-number of amino acid residues. This periodicity should thus be reflected in all of physicochemical and biological properties which are dependent upon the conformation and conformational stability of the molecules. Second, for GS derivatives with side-sequences of an odd-number of residues, polar and non polar residues will be segregated on opposite faces of the molecules (i.e. the molecules will be highly amphipathic) when the L-amino acids forming the side-sequences are arranged in an alternating sequence of polar and non polar residues. Consequently all physicochemical and biological characteristics of GS derivatives which are dependent upon their amphipathic characteristics will be affected by the structural manipulations which alter such properties. In the work presented here, we have examined some GS analogs of different ring sizes, and a number of analogs in which the peptide amphipathicity was changed by L to D substitutions of amino acids in the side-sequences. On the basis of work presented in this thesis and other work published elsewhere, various physicochemical attributes of GS-like antimicrobial peptides and their lipid membrane targets can be identified as being important to the expression of their antimicrobial and hemolytic activities [24, 25]. The main features examined are outlined below.

1. The size of the GS ring system

The data presented here and in other collaborative studies of this project [5, 8, 10 and unpublished data] all indicate that the size of the GS ring system strongly influences the antibiotic and hemolytic activities of these peptides and their general effects on various lipid membranes. The consensus and summation of all available experimental data on the effects of the size of the GS ring system on the overall membrane activity is illustrated in Figure 16.

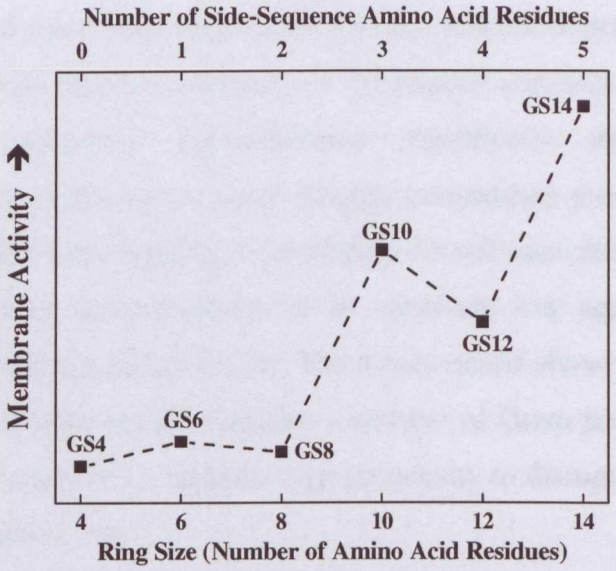

Figure 16. Effect of the size of the peptide ring system on the overall membrane activity of various ring size analogs of GS.

The pattern reveals that membrane activities (antibiotic, hemolytic and overall membrane disruptive activities) exhibited by GS analogs with small ring sizes (i.e.<10 amino acid residues) are relatively weak, but increase significantly in magnitude as the size of the GS ring system increases. This observation can be rationalized on the basis of sterical considerations, mainly because accommodating larger molecules in the polar/apolar interfaces of lipid bilayers should be inherently more disruptive on membrane integrity than would be the case with the smaller analogs. This suggestion may also explain why GS analogs such as GS12 and GS14*d*K_4 exhibit significant levels of antibiotic activity despite their poor partitioning into lipid membranes (see Results section). The idea that size *per se* may be an important component to the membrane activity of antibiotic peptides also implies that there may be a minimal size requirement for the expression of membrane activity. This possibility is supported by the results of our studies of GS analogs, and by studies of the membrane activity of some synthetic linear, α-helical forming, amphipathic peptides [51, 52, 53].

Another important factor related to the size of the peptide, arises from the fact that for any family of structurally related amphipathic peptides, an increase in peptide size will also be accompanied by an increase in hydrophobic surface area. Consequently, increases in size will probably be accompanied by an increased propensity for the penetration of and interaction with lipid membranes. Given this, the size-related increases in membrane activity observed by us and others may well be attributable to the combined effects of these membrane partitioning

considerations and the size-determined steric effects described above. However, with the increase in hydrophobic surface area there will also be an increase the peptide's propensity for self association in water, which, in turn, will decrease its aqueous solubility as a monomer. In principle, this latter consideration can pose very serious problems when one attempts to assay the membrane disruptive activities of such peptides. Especially in cases where the activity being measured is dependent upon the capacity to deliver the peptide to a membrane target via an aqueous medium (e.g, growth inhibition and membrane permeation assays). Indeed, depending upon the assay conditions, the low monomeric aqueous solubility of the peptide can become a very effective kinetic barrier against achieving thermodynamic equilibrium thereby underestimating the true membrane activity of the agent. Interestingly, temperature-profiling reversed phase HPLC studies indicate that GS14 has significant propensity for self association in aqueous media [54], consistent with previous demonstrations of its relatively low aqueous solubility and its tendency to aggregate in aqueous solution [24]. The issues raised above may, in part, explain why the assayed antibiotic activity of GS14 against a number of Gram positive and negative bacteria is very low [22, 26], despite of its innately high propensity to disrupt lipid membranes [see section 3.3. of this thesis and ref. 54].

An increase in membrane disruptive activity accompanying the increase in GS-ring size exhibits a distinct periodicity, based on whether the side-sequences contain an odd- or an even number of amino acid residues (Figure 16.). This behavior can be attributed to the aforementioned odd/even periodicity in the conformation and β-sheet content of GS-like cyclic peptides (see above) and in particular, to the effect of these factors on the facial amphiphilicity which directly effects their capacity to interact with lipid membranes [54].

2. *The amphipathicity of the GS ring system*

The amphipathicity of the GS ring system is probably the largest single factor affecting the antibiotic, hemolytic and overall membrane disruptive activities of these peptides. This fact has been confirmed by numerous studies, which indicated that the biological activities of GS-like peptides are all positively correlated with their amphipathic characteristics [22, 26]. As noted above, maximal amphipathicity is achieved within the GS structural motif, when the "side-sequences" are composed of an odd number of amino acid residues arranged in an alternating sequence of hydrophobic and cationic L-amino acid residues. This configuration induces the formation of a very stable antiparallel β-sheet which is also stereochemically favorable to stabilization by cross-ring hydrogen bonding (Figures 1 and 2). It also produces a highly amphipathic structure with hydrophobic and cationic side chains of the side-sequences projecting on opposite faces of the ring system (Figures 1 and 2). The requirements for

maximizing amphipathicity within the GS structural motif are thus highly specific, and intimately linked to structural factors such as conformation of the peptide, its β-sheet content, etc. Our studies of the effects of ring-size, of the enantiomeric inversions or essentially any structural modification of the GS ring system (aside from steric considerations of ring size, see above) on the hemolytic, antibiotic and other membrane disruptive activities of peptides, can be rationalized on how the various structural modifications affect the amphipathicity of the GS ring system. Our studies indicate that there is a distinct periodicity in the overall membrane activity, superimposed on the ring-size effects described above (see Figure 16). This periodicity is also apparent in the CD and ^{1}H-NMR spectroscopic data which show that β-sheet structure is retained in peptides GS6, GS10, and GS14, and is minimal with peptides such as GS4, GS8, GS12 [22, 28]. These observations all correlate nicely with the fact that the GS derivatives, GS6, GS10 and GS14 all fulfill the structural requirements for maximal amphipathicity (having side-sequences of an odd-number of residues i.e. 1, 3 and 5, respectively; see Figure 16), which are arranged in an alternating sequence of hydrophobic and cationic residues (i.e. K, VKL and VKLKV, respectively).

Considerations of the amphipathicity of the GS ring system can also explain most of the results obtained in studies of the effects of single and multiple enantiomeric inversions performed by us and others [23, 26, 55, 56]. The general consensus of the data obtained in such studies is as follows. First, enantiomeric inversions of the D-Phe-Pro residues which form the type II' β-turns result in a near complete loss in antibiotic and other membrane disruptive activities. Second, antibiotic activity and other manifestations of membrane activity are largely retained when single enantiomeric inversions of the hydrophobic residues are performed. Third, enantiomeric inversions of the cationic residues markedly diminish the manifestation of all forms of membrane disruptive activity, and this decline in activity increases markedly as the number of such inversions increase. These results can all be explained by an examination of the GS-ring system, and by considerations of the probable effects of enantiomeric inversions of the bridging, cationic and hydrophobic residues. Examination of the GS14 molecular models obviates that the presence of two antiparallel type II' β-turns is an absolute structural requirement for the formation and stability of its GS-like ring system. The loss of all structure, ring stability, amphipathicity etc. because of enantiomeric inversion of the turn-residues is thus not surprising and has been confirmed experimentally [26]. Molecular models also show that the C_α protons of the cationic side-sequence amino acid residues are all oriented inwards across the ring whereas those of the hydrophobic side-sequence residues all project outward from the side of the ring. Thus enantiomeric inversions of the cationic residues will be sterically unfavorable

because it will replace the inward-oriented C_α protons with charged bulky side chains. In turn this will be expected to destabilize the β-sheet conformation of the peptide and will also change the charge distribution on the surface of the molecule by projecting the cationic side chain amidst the hydrophobic side chains on the opposite surface of the molecule. In contrast, enantiomeric inversion of the hydrophobic residues should be more sterically favorable because the outward oriented C_α protons will be replaced with hydrophobic side chains. Molecular modeling studies also suggest that the change in the orientation of the hydrophobic side chains can be accommodated without drastic changes in the conformation, β-sheet content and capacity for cross-ring hydrogen-bonding. Given these observations, one can easily rationalize the large differences between the effects of enantiomeric inversions of the hydrophobic and cationic residues. The latter will have considerably greater deleterious effects on the amphipathicity of the GS ring system than would cause similar operations on the hydrophobic residues. Interestingly, because enantiomeric inversions of the cationic residues will also place a cationic side chain on the nonpolar face of the molecule [24, 25], it will also drastically alter the shape, the polarity and the properties of the hydrophobic face of the molecule. Most probably, this forms the basis of the diminished capacity of both membrane-partitioning and hydrophobically-driven self association in aqueous media as observed by us and others [24, 57]. The fact that the latter manipulations have a far greater influence on the manifestations of membrane activity is one of the factors that could be considered in part of rational design strategy (see below).

3. Peptide orientation and location within lipid bilayers

When GS penetrates phospholipid bilayers, it partitions into the polar/apolar interfacial region, with its ring plane parallel to the bilayer surface [19]. The hydrophobic side chains of the peptide structure interact with the lipid hydrocarbon chains and the positively charged cationic side chains interact with the lipid polar headgroups and water [19]. This arrangement is consistent with its amphipathic character and with the results of solid-state NMR studies of a fluorinated GS derivative [19]. Given the most probable location and orientation of GS-like peptides in lipid bilayers, a close examination of the structural properties of peptides which conform to the GS structural motif, can provide some clues to the probable basis of the membrane-disruptive activities of these peptides. For example, when a peptide such as GS14 partitions into the polar/apolar interfaces of lipid bilayers in its predicted most favorable orientation, it will probably laterally displace 6-8 lipid molecules [54]. However, because of the considerable mismatch between its hydrophobic length and the hydrophobic thickness of the host monolayer, there will be considerable packing and curvature strain on the membrane. The hydrophobic volume of the peptide would be insufficient to compensate for the combined

hydrophobic volumes of the lipid molecules displaced. Given these simple geometrical considerations, one would expect that the accumulation of significant amounts of GS-like peptides in any cell membrane will be inherently incompatible with the maintenance of a stable bilayer with viable barrier function. Furthermore, the inherent membrane disruptive capacity of any GS-like peptides will be dependent upon the average number of lipids that it laterally displaces upon insertion into the membrane. These expectations are consistent with the positive correlations found between membrane partitioning propensity and membrane activity [11, 12], and with the observation that membrane disruptive activity tends to increase as GS-ring size increases (see above). Moreover, the geometric considerations presented above may even form part of the basis of the tendency of these peptides to induce the formation of cubic phases in some types of lipid bilayers [11, 18, 20].

4. Peptide affinity for bacterial cell wall components

Bacteria possess either a lipopeptidoglycan outer barrier or a LPS-containing cell wall or outer membrane, respectively, both of which are lacking in organisms such as *A. laidlawii* B. LPS acts as a permeation barrier, rendering bacteria resistant to certain antibiotics. It has been suggested that the weak antimicrobial activity of GS14 against Gram negative bacteria may be attributable to its strong binding to the surface components of the bacterial cell wall, preferentially to the negatively charged lipopolysaccharides. LPS effectively competes for the binding of available peptide with the lipids of the cytoplasmic membrane, presumably by impeding the subsequent movement of the peptide, which means that GS14 is not reaching its target [24, 25]. This suggestion is supported by studies showing that the affinity of GS14 for LPS is at least an order of magnitude greater than those of the other analogs examined [26], and by the fact that GS14 exhibits a very high capacity to permeabilize model lipid membranes and to inhibit the growth the cell wall-less bacterium *A. laidlawii* B. The possibility that interaction of membrane-targeted antimicrobial peptides and other antimicrobial agents with negatively charged components of bacterial cell walls may essentially mask their inherent capacity to perturb bacterial membranes is not usually considered in most studies. Our studies suggest that this effect may also be accentuated by the fact that GS14 has a high propensity for self association in aqueous media (see above).

5. Differential peptide interactions with different classes of membrane lipids

A common feature of most membrane-targeted antimicrobial peptides is that their interactions with both model and biological membranes vary markedly with the lipid composition of their membrane targets. This aspect of the membrane interactions of GS (and many other types of antimicrobial peptides) has been widely studied by us and others [4, 12, 37,

52], to examine whether these differences may constitute a sufficient basis for the capacity to discriminate between bacterial and animal cell membranes. The overall consensus of such studies is that, consistent with their cationic character, antimicrobial peptides such as GS interact very strongly with anionic lipid membranes, less strongly with zwitterionic and nonionic membranes and very poorly with cationic lipid membranes. The fact that these peptides interact most strongly with negatively charged lipid membranes suggests that negative surface charge may be an important aspect of their activity, in vivo. However, one should note that these peptides are capable of interacting with nonionic and even cationic lipid membranes, and that they can quite efficiently permeabilize zwitterionic lipid vesicles at concentrations comparable to (though always lower than) those needed to permeabilize anionic lipid vesicles [12]. These observations suggest that negative surface charge *per se*, may not be a necessary requirement for the interaction of these peptides with lipid membranes, though it facilitates such interactions. In turn this suggests that interactions between the hydrophobic surfaces of such peptides with lipid membranes may be the dominant factors in their interactions with and penetration of lipid membranes [23, 25].

6. *Peptide affinity for fluid/disordered lipid membranes*

Membrane fluidity and/or disorder seems to be an important requirement for effective penetration by GS-like peptides, a feature which may well be applicable to other classes of membrane active amphipathic peptides [4, 7]. GS-like peptides interact more strongly with disordered membranes such as PC than with PE membranes which, form more ordered bilayer structures however their interactions were weak with cholesterol-rich lipid membranes [12, 13]. The high affinity of these peptides for fluid and/or disordered lipid membranes can be explained by the observations that their binding to and/or penetration of lipid membranes is an entropy-driven process against which considerable enthalpic work is expended [57]. The high affinity of these peptides, and indeed many other types of antimicrobial peptides, for disordered lipid membranes may also explain the tendency for cholesterol to attenuate the potentially deleterious effects of GS and other membrane-disruptive peptides [17]. The low affinity of these (and possibly other) peptides for cholesterol-rich membranes may be largely responsible for their capacity to differentiate between target membranes (see below).

Discrimination between bacterial and eukaryotic membranes

Understanding the governing factors in target selectivity, delineating the features determining high antimicrobial activity coupled with low hemolytic activity are crucial for rational AMP- based drug development. In a previous study the GS-based synthetic analog,

GS14*d*K$_4$ was found to exhibit an enhanced capacity to discriminate between bacterial cells and erythrocytes [26]. We have therefore used FTIR spectroscopy to study the interaction of this peptide with single- and multicomponent membranes and to understand the molecular basis of its increased selectivity for bacterial membranes (see Results sections 3.6. and 3.7.). A comparison of these results with those of comparable studies of GS, which exhibits a considerably weaker capacity for discriminating between bacterial and mammalian cell membranes [17, 18] reveals a number of significant and potentially relevant features. First, as observed with GS, the degree and extent of the GS14*d*K$_4$ interaction varied with the nature of the lipid bilayers examined, suggesting that the composition of the lipid membrane is an important aspect to the selectivity parameters exhibited by both peptides. Second, at comparable lipid:peptide ratios, the effects of GS14*d*K$_4$ on all classes of lipid bilayers were considerably weaker than those of GS, suggesting that GS14*d*K$_4$ is either inherently less perturbing to lipid bilayers and/or that the partitioning of GS14*d*K$_4$ into lipid bilayers under our experimental conditions is considerable less favorable than that of GS. Third, and most significantly, our results indicate that GS14*d*K$_4$ interactions with cholesterol-rich PC bilayers are very weak and suggest that GS14*d*K$_4$ is essentially excluded from such membranes. This result contrasts sharply with those obtained in our studies of GS showing significant partitioning of GS into cholesterol-rich PC bilayers, albeit at markedly lower levels than observed with cholesterol-free PC bilayers. Given that the outer lipid surfaces of animal cell membranes are essentially cholesterol-rich zwitterionic lipid monolayers [29], and the fact that bacterial membranes are essentially devoid of cholesterol [30], suggest that the poor solubility of GS14*d*K$_4$ in cholesterol-rich zwitterionic lipid membranes may actually be the largest single contributing factor to its weak hemolytic activity. This suggestion is supported by the results of recent isothermal titration calorimetric studies [57]. In fact it looks like the major differences between the two types of membranes make bacterial membranes more susceptible to the action of antimicrobial peptides such as GS, than animal cell membranes. Bacterial cells usually carry an inside negative transmembrane potential which is often considerably larger than that of animal cells [29, 31]. Lipid membranes at the surfaces of bacterial cells lack cholesterol [29, 30] but contain substantial amounts of negatively charged phospholipids which may facilitate interaction with negatively-charged phospholipids. Our results suggest that the above described differences between the lipid compositions as well as between the outer membrane surfaces of bacterial and animal cells probably form the physical basis of the mechanism whereby antimicrobial peptides such as GS can discriminate between the two types of cells. It is also interesting to note that, although GS is considerably more perturbing of lipid bilayers than

GS14*d*K$_4$, at comparable lipid:peptide ratios, the latter peptide retains considerable antimicrobial activity which, in some cases, is comparable to that of GS itself [26]. Given the lower GS14*d*K$_4$ partitioning into lipid bilayers, and the evidence that it probably destabilizes membranes by a similar mechanism to GS [54], it follows that GS14*d*K$_4$ must be inherently more disruptive to lipid bilayers than GS. Most probably, this can be attributed to the larger size of the GS14*d*K$_4$ molecule (see above).

New findings

1. Within the context of the GS structural motif, peptides with larger ring-sizes are intrinsically more disruptive of membrane cohesion once they are inserted into the polar/apolar interfacial regions of lipid bilayers.

2. Despite of a high intrinsic lytic activity, the weak antimicrobial activity of GS14 against Gram negative and positive bacteria could be a reflection of its restricted access to the cell membranes of these microorganisms due to its preferential binding to bacterial cell surface moieties such as LPS or peptidoglycans, and/or its aggregating in aqueous media.

3. Within any group of structurally related GS-like peptides, the highest therapeutic indices are usually not observed with those peptides which exhibit the highest facial amphilicities, the highest membrane partitioning propensities, nor the highest antibiotic activities. This suggests that there is an optimal range of peptide amphiphilicity which is favorable for achieving high selectivity.

4. Cholesterol reduces the membrane affinity and penetration of antimicrobial peptides presumably because it increases membrane cohesion by decreasing overall membrane fluidity and decreasing the conformational disorder of liquid-crystalline lipid hydrocarbon chains.

5. At comparable lipid:peptide ratios, GS14*d*K$_4$ is considerably less disruptive of lipid membranes than GS. Nevertheless, it exhibits a considerably higher capacity to lyse bacterial cells in preference to red blood cells. These properties can be attributed to the combination of its higher innate tendency to disrupt lipid bilayers once inserted (because of its larger size), its preferential interactions with the anionic lipids present on the surfaces of bacterial membranes, and its low affinity for the cholesterol-rich zwitterionic lipid monolayers found at the surfaces of animal cells.

Conclusions

The mode of interaction of various cyclic cationic peptides with conformational restrictions and flexibility, differences in size, structure, amphipathicity, hydrophobicity, distribution of positive charges on the surface, with biological membranes is complex. Their

biological effect is a result of a unique combination of their physical properties in aqueous and membrane environments, and depends on the lipid composition and fluidity of the targeted membrane as well. Owing to the diversity of the above described physical properties of peptides and lipid bilayers of biological membranes, the structure-function relationship of the cyclic cationic peptides has a complex nature and cannot be understood solely on the basis of the biophysical properties of the peptides without considering the mutual interaction with other components of the biological environment [25]. Nevertheless, there are some drug design principles that have emerged from our work on GS analog peptides and from recent follow up studies [56, 57] to guide future drug development decisions.

1. As long as the GS conformational motif is maintained or not drastically distorted, increases in ring size result in an increase in the innate capacity of the peptide to disrupt cell membranes. The bigger the molecule the more damage it is likely to do if it penetrates the membrane. Thus increasing ring size may be a viable option for engineering higher antibiotic activity. However, increasing ring size is likely to increase the propensity for self association in aqueous media, a factor which may set an upper limit to the viability of this option.

2. Structural modifications to the nonpolar surfaces of GS-like derivatives have a considerably greater effect on overall membrane activity than do changes to the polar surface. Presumably because the polar surface of the peptides is the one that actually penetrates the polar/apolar interfaces of lipid membranes and ultimately determines how much of the peptide can partition into the membrane. It thus follows that rational fine tuning of peptide selectivity and antibiotic effectiveness may be best achieved by rational modifications of the nonpolar surfaces of these molecules.

3. High antibiotic activity based on a high innate capacity to partition into cell membranes is probably inherently incompatible with the capacity for effective discrimination between bacterial and animal cell membranes. Rational design of the high selectivity required for therapeutic usefulness may thus require some sacrifice in the antibiotic effectiveness coincident with a high innate capacity for partitioning into lipid membranes.

4. Interactions between these cationic antimicrobial peptides with the negatively charged lipids of bacterial membranes contribute to the capacity for bacterial selectivity, but themselves are probably not enough to render the peptides therapeutically useful.

5. Poor partitioning of these peptides into cholesterol-rich zwitterionic lipid membranes is probably the biggest single factor underlying the capacity of these peptides to discriminate between bacterial and animal cell membranes. This fact should be considered in the

development of *in-vitro* models for estimating the actual selectivity of these peptides towards bacterial cell membranes.

These basic design concepts follow naturally from general considerations of the physical processes underlying the partitioning of peptides into lipid membranes. Thus in principle, they should be applicable to other types of antimicrobial peptides. Indeed, one of these concepts (see #2 above) has recently been applied to in the rational design of some α-helical antimicrobial peptides with enhanced selectivity for bacterial cells over erythrocytes [56].

Finally, although a substantial amount of biophysical, physicochemical and biological data is available concerning the interactions of GS and its peptide analogs with lipid model and biological membranes much remains to be done. In particular, studies of the affinity and thermodynamic profile of peptide binding in the bilayer, as well as on the actual mechanism of action are required, to construct a detailed model of the mode of action of cyclic cationic peptides on different phospholipid bilayers. Experiments are currently underway to determine the phospholipid bilayer/water partition coefficient, the localization and orientation of antimicrobial peptides in the phospholipid bilayer, and the effects of these peptides on phospholipid organization and packing. Approximation of the real membrane composition with artificial mixtures and the examination of the interactions of GS-like peptides with such complex bilayers would also be beneficial. One of the current problems in interpreting virtually all work on the interaction of antimicrobial peptides with lipid membranes is that it is not really known how much of the peptide partitions into the membrane under the conditions of the experiment, which is one of the areas that need to be addressed in the future. Further studies are needed, like a recent isothermal titration calorimetry study [57] in which the degree of binding of GS14*d*K_4 to various types of lipid bilayers has been determined, moreover it gave a comprehensive thermodynamic description of the binding process. These results were particularly important as they allowed the determination of the amount of peptide actually present in the membrane as opposed to the amount of added peptide in the aqueous solution. These results should allow us to quantitate various parameters and gain additional insight into the molecular basis of the structure/activity correlations reported in this thesis.

Our results indicate that studies of the interactions of GS analogs with phospholipid vesicles are useful for predicting their hemolytic nature and their antimicrobial potency. These investigations contribute to the understanding of the molecular basis for their differential and/or preferential antimicrobial potencies against different classes and species of bacteria, many of which differ considerably in the lipid compositions of their membranes.

5. Acknowledgement

I gratefully thank the Hungarian Ministry of Education for a three-year PhD fellowship and the Eötvös József Foundation for a fellowship to the University of Alberta. This work was supported by the Hungarian National Science Fund (OTKAT029458), the Volkswagen-Stiftung, grants from the Canadian Institute for Health Research, the National Institute for Health (NIH: R01 GM061885 RSH), the Alberta Heritage Foundation for Medical Research and the Protein Engineering Network of Centers of Excellence of the University of Alberta.

I greatly acknowledge to Prof. László Dux and Prof. Ronald N. McElhaney for providing the opportunity to work at their departments and laboratories, for their personal and financial support and for the supervision and scientific guidance in various research areas and projects I have been involved.

I am especially and mostly grateful to Dr. Elmar J. Prenner, my supervisor, tutor and friend for his instructions, scientific guidance and especially for his patience, for always encouraging and supporting me over the past years.
I am equally thankful to Dr. Ruthven N.A.H. Lewis for his co-supervision, technical and scientific guidance, for his friendship and for the many constructive discussions and suggestions he gave me without sparing his time and energy.

I am obliged to Dr. Tibor Páli for introducing me the antimicrobial peptide research field, for the teaching, the scientific guidance and friendship, for his valuable advices and help in preparing this Thesis.

I am especially thankful to my husband Dr. Tamás Csont, without him I have not been able to face all the challenges I had to. I would like to express my special gratitude to my parents Prof. and Mrs. Imre Kiricsi, my little son Botond Csont, my sister Ágnes and the Csont family for their never-ending love and support.

I am also very grateful to my colleagues for the friendly and constructive atmosphere, to all my teachers and supervisors for their guidance and instructions and to all my friends for their love and support.

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7. Annex

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