Investigations of the synergistic enhancement of antimicrobial activity in mixtures of magainin 2 and PGLa

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Abstract:

Magainins are antimicrobial peptides isolated from the African clawed frog *Xenopus laevis*. They interact with bacterial membranes where they cause channel formation and membrane disruption. When added as a cocktail magainin 2 and PGLa are considerably more efficient when compared to the corresponding amounts of individual components. In order to investigate this synergistic interaction of PGLa and magainin a number of magainin variants have been prepared and investigated in biological and biophysical assays. In particular we report on the antimicrobial activities and solid-state NMR investigations of magainins that have been extended by a carboxyterminal GGC tripeptide to form covalently linked dimers. Notably, when the formation of the covalent linkage is prevented by exchanging the cysteine by serine or alanine no loss in efficiency was observed indicating that the covalent interaction is not necessary for synergistic interaction. In a next step peptides labelled with ^{15}N and ^2H were reconstituted into oriented membranes and their topology studied by solid-state NMR spectroscopy. The tendency of some of these peptides to adopt membrane-spanning alignments does not correlate with their synergistic activities in antimicrobial assays. In contrast, the stable alignment of PGLa parallel to the surface of membranes made of *E. coli* lipid extracts is strongly suggestive that the peptides develop synergistic activities when in an in-planar configuration. Notably, the phospholipid head groups these samples show a high degree of disturbance suggesting that the synergistic interactions between the magainin peptides could be mediated through the lipid phase.

LIST OF ABBREVIATIONS

CI: combination index

di-C10:0-PC: 1, 2-didecanoyl-*sn*-glycero-3-phosphocholine

DMPC: 1, 2-dimyristoyl-*sn*-glycero-3-phosphocholine

DMPG: 1, 2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)

ITC: Isothermal titration calorimetry

MIC_n: minimal concentration to inhibit n% of bacterial growth

MH: Mueller Hinton

NBD: 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole

NMR: nuclear magnetic resonance

OD: optical density

PC: phosphatidylcholine

PE: phosphatidylethanolamine

PG: phosphatidylglycerol

POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

POPE: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine

POPG: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)

RET: resonance energy transfer

r.h.: relative humidity

The spread of multi-resistant pathogens has become an increasing threat to human health where immediate actions are needed. Bacteriocidal and fungicidal molecules are abundant in nature and promise that new pharmaceuticals can be developed by mimicking the mechanism of action of such antimicrobial compounds [1-3]. Host defense peptides are one class of antimicrobial macromolecules that have indeed served as lead compounds for the creation of more easy to prepare and/or more efficient analogues [4, 5]. Whereas in some organisms they are over-expressed when infections occur, such peptides are also stored in exposed tissues of animals and plants, where they provide an even faster and highly efficient response [1-3]. Such peptides are wide spread and currently the data bases contain over 2500 sequences from various organisms and by design [6]. A good number of peptides have been identified from frogs and other amphibians and were among the first peptides to be described for their antimicrobial activities [7, 8]. These linear peptides are characterized by hydrophobic and a great number of cationic residues which confer a highly amphipathic character. When tested in biological assays they exhibit a broad spectrum of antimicrobial activities. Virucidal and tumoricidal characteristics have also been detected for magainins and other host defence peptides [9, 10].

When magainin peptides associate with phospholipid membranes they have been shown to adopt amphipathic α -helical conformations with the cationic residues accumulating on one face of the helix and hydrophobic residues opposite [11-13]. When the helical domain intercalates into the membrane interface it acts like a wedge thereby destabilizing the membrane packaging [14]. As a consequence the phospholipid bilayers become leaky, the electric conductance increases and the transmembrane electrochemical gradients collapse, which ultimately results in cell killing by these and other amphipathic peptides (reviewed in e.g. [4, 15]). The notion that membranes rather than proteinaceous receptor are the main targets of these peptides is supported by observations that enantiomers, retromers or retroenantiomers of magainins and cecropins from insects, all exhibit the high antibiotic and pore-forming activities of the parent L-peptides. [16]. Once the peptides have crossed the membrane barrier intracellular targets may also become important for some peptides (for a review see [17]).

A number of biophysical investigations have been performed with magainins [12, 18-22], magainin analogues [23-25] and related cationic antimicrobial peptides [26, 27] which revealed interesting details about their interactions with membranes. These studies revealed that the peptides preferentially intercalate into the bilayers at orientations parallel to the membrane surface in agreement with their strongly amphipathic and cationic character [4]. Notably, such biophysical data have been shown to correlate well with their activities in antibacterial assays [28], where the lipid-dependent membrane interactions of the aurein frog peptides parallels nicely their activities against bacterial species in biological assays [29]. Therefore, when insights from biophysical investigations where combined with structure-activity correlations (e.g. [30]) a number of mechanisms of action were proposed to describe pore formation in membranes either *in vitro* or in bacteria [4]. Such models include the formation of toroidal pores, where the peptides together with the lipid form a supramolecular assembly of high curvature [31, 32]. Furthermore the 'carpet model' was suggested, where the peptides crowd at the membrane at an alignment parallel to the surface. [15]. When a critical peptide concentration is reached the bilayer disintegrates. At lower peptide concentrations stochastic fluctuations of the peptide density along the membrane interface occur and can explain the transient and step-wise increases in membrane conductivity that have been observed experimentally [4]. Atomistic views supporting such models have also been obtained by molecular modelling calculations [33]. The various supramolecular arrangements of peptides and lipids have recently been summarized in a phase diagram that takes into account that Soft Membranes Adapt and Respond, also Transiently, to external stimuli (SMART model) [34]. Within this model one supramolecular assembly can transform into another shape and this depends on a variety of factors such as the peptide-to-lipid ratio, lipid composition, temperature, buffer composition and pH or other environmental factors. Within the phase diagram of the SMART model the peptides can cause lysis, formation of transient channels and under some conditions even increase the stability of lipid bilayers [14]. Having understood that peptides located at the membrane interface can exhibit strong antimicrobial activity without adopting transmembrane orientations, has resulted in the design of very

short sequences or of peptide mimetics with considerable antibiotic action [35-39].

In this paper we follow up an interesting additional observation namely the synergistic enhancements of activities of antimicrobial peptides when added in combination, which also occurs in cocktails of magainin 2 and PGLa, two peptides that are produced and stored together in the frog skin. Notably the peptides' activities are increased not only in biological assays but also when the release of fluorophores from model membranes is monitored. Indeed the addition of equimolar mixtures of these two peptides causes an increase of efficiency by almost an order of magnitude when compared to the individual components. Synergism has also been observed for other amphipathic peptides as well as for mixtures involving conventional antibiotics [13, 40-43].

So far only a few biophysical or structural investigations have been published and the molecular interactions that cause synergistic enhancements are poorly understood. Using ^{15}N solid-state NMR spectroscopy the membrane alignment of PGLa and magainin 2 was monitored. In phosphatidylcholine membranes, where both fatty acyl are saturated, the tendency of PGLa to adopt transmembrane alignments increased in particular in the presence of magainin 2, when at the same time the latter remains surface oriented [44, 45]. Importantly, both peptides maintain orientations parallel to the membrane surface in bilayers made of (partially) unsaturated phospholipids (e.g. POPC or POPC/POPG). Notably the latter are thought to represent more closely the natural composition of biological membranes and this result therefore seems to represent well what happens in a natural environment [45, 46].

Previously PGLa and magainin 2 variants were prepared with GGC extensions at either the carboxy- or the amino terminus as well as protecting groups neutralizing the charges of both termini [47]. Mixtures of these sequences were incubated in the presence and absence of PC/PG vesicles under high pH conditions favouring cystine bridge formation. Notably, when associated with membranes the parallel heterodimer of the two peptides preferentially formed [47]. This preformed dimer was found equally active than the mixture of wild type peptides when tested against bacteria, when at the same time the covalent linkage much

enhanced calcein release activity from egg-PC vesicles [48]. Although such data are suggestive that specific interactions between PGLa and magainin 2 are responsible for their synergistic activities it has proven difficult to identify unique and strong interaction sites on both peptides [41, 49]. Therefore, we further investigated the role of sequence additions that have been suspected to help in dimer formation and thereby enhance synergistic activities [47] as well as variants thereof. These sequences were tested in antimicrobial assays and the data compared with their tendency to adopt transmembrane alignments investigated. Because the in-plane to transmembrane equilibrium of PGLa is most easily shifted into its transmembrane state in thin membranes investigating ^{15}N labelled PGLa in di-C10:0-PC by ^{15}N solid-state NMR spectroscopy provides a sensitive test for such shifts in topological equilibria. [45]. Therefore, we tested how PGLa and the modified PGLa sequences align in such thin membranes in the absence and presence of magainin 2 peptides and how their tendency to adopt more transmembrane alignment correlates with their potential to interact synergistically.

MATERIALS AND METHODS

The phospholipids (di-C10:0-PC, *E. coli* lipid extract) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification.

Peptide synthesis and purification

The peptides sequences shown in Table 1 were prepared by solid-state synthesis using a Millipore 9050 automatic peptide synthesizer and fmoc chemistry. For NMR investigations PGLa was labelled with ^{15}N at the Ala-14 and with $^2\text{H}_3$ at the Ala-10 position using commercial fmoc-protected amino acids (Euriso-top, Paris, France or Isotec® Sigma-Aldich St Quentin Fallavier, France).

The peptides were purified by reverse phase HPLC (Gilson, Villiers-le-Bel, France) using a preparative C18 column (Luna, C18-300Å-5µm, Phenomenex, Le Pecq, France) and an acetonitrile/water gradient. Their identity and purity (> 90%) were checked by analytical HPLC and MALDI mass spectrometry (MALDI-TOF Autoflex, Bruker Daltonics, Bremen, Germany). The purified peptide was lyophilized and stored at -20°C.

Table 1: Peptide sequences

(-NH₂ is indicative of an amidated, -COOH of a free carboxy terminus)

PGLa	GMASK AGAIA GKIAK VALKA L-NH ₂
PGLa (L18W)	GMASK AGAIA GKIAK VAWKA L-NH ₂
PGLa-GGC (L18W)	GMASK AGAIA GKIAK VAWKA LGGC-NH ₂
PGLa-GGS (L18W)	GMASK AGAIA GKIAK VAWKA LGGS-NH ₂
PGLa-GGA (L18W)	GMASK AGAIA GKIAK VAWKA LGGA-NH ₂
Magainin 2	GIGKF LHS AK KFGKA FVGEI MNS-NH ₂
Magainin 2-COOH	GIGKF LHS AK KFGKA FVGEI MNS-COOH
Magainin 2-GGC	GIGKF LHS AK KFGKA FVGEI MNSGG C-NH ₂
Magainin 2-GGS	GIGKF LHS AK KFGKA FVGEI MNSGG S-NH ₂
Magainin 2 (scr, F5W)	GIGKW LHS AK KFGKA F GEIMINS-NH ₂

Determination of antimicrobial activities

For antimicrobial assays *E. coli* bacteria (ATCC25922, Ref 0335-CRM, Thermo Fisher Scientific, Courtaboeuf, France) were grown overnight on Mueller-Hinton (MH) agar plates and suspended in MH media (Fluka Analytical, Sigma Aldrich, Saint-Louis, MO, USA) to inoculate a 10 ml preculture. The preculture was grown overnight (no longer than 18 h), and used for the inoculation of test cultures (10 ml of MH) at an OD₅₅₀ of 0.2. The test cultures were grown for 3-4 h at 37 °C at 200 rpm, the turbidity was again adjusted to an OD₅₅₀ of 0.2 and from this, a 1000-fold diluted bacterial suspension was obtained corresponding to 1- 2 · 10⁶ CFU/ml (colony forming units).

The antimicrobial assay was performed in 96 well microplates (F-bottom sterile non treated polystyrene, Nunc, Thermo Scientific Nunc A/S, Roskilde, Denmark). The first column is loaded with 50 µl of doubly concentrated MH (2X) and completed with 50 µl of an 800 µM solution of peptides in 50% ethanol. Columns 2 to 11 are loaded with 50 µl 1X MH. A two-fold dilution is successively performed from column 1 to 10 containing 50 µl of 1X MH 1X. Finally, the previously prepared bacterial suspension was distributed by adding 50 µl to each

well (except column 12) resulting in $5 \cdot 10^4$ and $1 \cdot 10^5$ CFU/well. The final peptide concentration ranges from 200 μM to 0.391 μM (columns 1 to 10). In each series, controls for bacterial growth (without peptides, wells of column 11), for contamination (without bacteria, column 12). Another control consisted in an additional row with the same two fold dilution than for peptides but where the ampicillin final concentration ranges from 25 to 0.0488 μM (positive control for killing bacteria. Each condition was performed as triplicate within each series. Microplates are incubated at 37°C for 22 h without stirring in the incubator (Mettler INB-400, Mettler GmbH+Co.KG, Schwabach, Germany).

To monitor the bacterial growth and to determinate the MIC₁₀₀ (complete inhibition of bacterial growth) of each peptide or peptide mixture, a colorimetric assay was used [50]. Resazurin when oxidized is blue, non-fluorescent and absorbs at 610 nm. When reduced, it turns into resofurin which is pink, fluorescent and absorbs light at 610 nm. Growing bacteria release molecules from their respiratory cycle which reduce resazurin, turning the blue bacterial suspension pink. Therefore, 20 μl of resazurin at 0.2 mg/ml were added to each well and the microplates were incubated for an additional 2h at 37°C. The colour change was observed by eye, and monitored spectroscopically. To determine the MIC, the difference between OD₅₇₀ and OD₆₀₀ is calculated after background subtraction and averaging of triplicates.

An additional microplate regrouping the assay of different peptides was prepared and placed into the microplate reader (MultiSkan Go, Thermo Fisher Scientific, Courtaboeuf, France) with incubation at 37°C for 22h under shaking at low speed. The OD₆₀₀ was measured every 30 min. The two methods (colorimetric and by difference of absorbance at 570nm and 600nm) were compared to each other to determine the MIC₁₀₀.

For most of the experiments, the commercial Mueller Hinton media has been prepared and autoclaved without further modification. When checked with a pH electrode, a value of 8.5 was measured in some preparations. Therefore, some experiments were repeated at a lower pH of 7.2 or 5.49 by adding sterile HCl to the autoclaved media.

Evaluation of synergistic activity

To evaluate the synergistic activities the optical measurements were analysed in quantitative detail by first normalizing the dose response curve and then applying

a sigmoidal fit of the form: $y = \frac{1}{1 + \left(\frac{x}{MIC_{50}}\right)^m}$, where x is the total peptide

concentration, MIC_{50} is the concentration inhibiting 50% of the bacterial growth ($y = 0.5$ at MIC_{50}) and m is the slope which has been fixed to 20 for this data analysis.

The MIC_{50} determined in this manner for the peptides individually and for the peptide mixtures was then compared to the expected value in the absence of synergism with the experimental one. The expected curve for the mixture of peptide in the absence of synergism is calculated by considering the MIC_{50} of each peptide alone with equal contribution as they are at molar ratio 1:1 by the

following equation: $y = \frac{1}{1 + \left(\frac{0.5x}{MIC_{50}^a} + \frac{0.5x}{MIC_{50}^b}\right)^m}$ (with x being the total peptide

concentration). The thus calculated expected MIC_{50} of the mixture was then compared to the one obtained by experiment.

The combination factor CI is then calculated from the MIC_{50} of each peptide alone and of the mixture using the following equation [51]:

$$CI = \frac{0.5 \cdot MIC_{50}^{a+b}}{MIC_{50}^a} + \frac{0.5 \cdot MIC_{50}^{a+b}}{MIC_{50}^b}. \text{ A CI value of 1 is indicative of additivity, } CI > 1 \text{ of}$$

antagonism and $CI < 1$ of synergism. The synergistic enhancement is obtained as $1/CI$.

Sample Preparation for solid-state NMR measurements

A homogeneous mixture of lipid and peptide in chloroform/methanol (1:1 by volume) was spread onto ultra thin cover glasses (8×22 mm, thickness 00, Marienfeld, Lauda-Königshofen, Germany), dried first in air and thereafter under vacuum overnight. Thereafter the membranes were equilibrated at 93% relative humidity before the glass slides were stacked on top of each other and sealed in plastic wrapping [52].

Solid-state NMR Spectroscopy

Proton-decoupled ^{15}N cross-polarization (CP) spectra were acquired on a Bruker Avance wide-bore 400 MHz NMR spectrometer equipped with a double resonance flat-coil probe [53]. An adiabatic CP pulse sequence was used [54] with a spectral width, acquisition time, CP contact time, and recycle delay of 38.5 kHz, 6.7 ms, 0.8 ms, and 3 s, respectively. The ^1H $\pi/2$ pulse and spinal 64 heteronuclear decoupling field strengths were 35 kHz. Typically 40000 scans were accumulated, and the spectra were zero filled to 4096 points. Typically, a 200 Hz exponential line-broadening was applied before Fourier transformation. Spectra were externally referenced to $^{15}\text{NH}_4\text{Cl}$ at 40.0 ppm [55].

Thereafter the baseline was corrected between -30 ppm and -470 ppm. The ratio of the transmembrane signal intensity was calculated by comparing the integral from 270 ppm to 150 ppm with that obtained between 270 ppm and 30 ppm.

Deuterium solid-state NMR spectra were recorded using a quadrupolar echo pulse sequence [56] with a spectral width, acquisition time, interpulse delay, and recycle delay of 500 kHz, 8.2 ms, 50 μs , and 1.5 s, respectively. The spectra were referenced relative to $^2\text{H}_2\text{O}$ (0 Hz). An exponential apodization function corresponding to a line broadening of 300 Hz was applied before Fourier transformation.

Proton-decoupled ^{31}P solid-state NMR spectra were acquired at 161.953 MHz on a Bruker Avance widebore 400 NMR spectrometer equipped with a double resonance flat-coil probe [53]. A phase cycled Hahn-echo pulse sequence [57] with a $\pi/2$ pulse of 2.5 μs , a spectral width of 75 kHz, an echo delay of 40 μs , and a recycle delay of 3 s was used. Spectra were referenced externally to 85% H_3PO_4 at 0 ppm.

RESULTS

Antimicrobial Assays

When Magainin 2 amide and PGLa were tested for antimicrobial action against *E. coli*. MIC₁₀₀ values of 25 μM - 12,5 μM were obtained. When the two peptides were

added as an equimolar mixture, the total peptide concentration needed to inhibit bacterial growth was considerably reduced in agreement with previous publications [40, 41]. Performing 2-fold dilution series, measuring the optical density or judging the redox potential in the presence of reazurin are wide-spread methods and the corresponding MIC₁₀₀ values of magainin, PGLa and of a number of variants investigated here are presented in Table 2. In order to quantitatively evaluate the synergistic enhancement of such mixture we used a more refined method where the growth curves were statistically analysed and fitted by sigmoidal transitions (Figure 1). This approach allows one to evaluate the MIC₅₀ and its standard deviation (SD) more accurately. Notably, the MIC₅₀ values determined here are about two to three times reduced when compared to the MIC₁₀₀ obtained with the colorimetric method (Table S1).

Figure 1: Normalized growth inhibition as a function of PGLa_L18W (A) and magainin 2-NH₂ concentration (B). The black line is obtained by fitting the experimental data set of triplicates represented by its averages and standard deviation. The MIC₅₀ is also indicated.

The MIC₅₀ values for each peptide and their mixtures were thus determined (Figure 1). In a next step the curve (MIC₅₀) that is expected when two peptides are mixed in the absence of synergism is calculated and compared to the experimentally measured growth inhibition (Fig. 2). From this comparison a combination factor (CI), or the synergistic enhancement is calculated.

Figure 2: Quantitative analysis of the synergistic enhancements of A. PGLa L18W and magainin 2, B. PGLa L18W and magainin 2-GGC and C. PGLa L18W and magainin 2-GGS. From the normalized fits (cf. Figure 1) for PGLa (dashed line) and magainin 2-NH₂ (dotted line), the expected transition curve for the corresponding mixtures of PGLa and magainin 2 peptides at molar ratio (1:1) and in absence of synergism is calculated (red line) and compared to the experimental data for the mixture (black square) and its fitted transition (green line.)

In the past a number of peptide variants and combinations have been tested in biophysical experiments and antimicrobial assays. When the PGLa sequence was modified by replacing L18 by tryptophan, the resulting sequence was equally active in fluorescence dye release assays and synergistic activity with magainin 2 [41]. Here, we demonstrated that this variant exhibits the same antimicrobial activity as unmodified PGLa (Table S1).

Furthermore, the synergistic activities of PGLa and of magainin 2 extended by GGC at the carboxy-terminus were tested. This modification had been introduced previously to study the effect of dimerization on the biophysical characteristics of these peptides [47]. When added individually, PGLa-GGC (L18W) and magainin-GGC are both 2- to 3-times more active in the antimicrobial assays when compared to PGLa (L18W) and magainin 2 alone, respectively (Table 2). When the cysteine is replaced by serine or alanine, this improvement is reverted and MICs that are the same range or higher as those of the sequences without tripeptide extension are obtained (Table 2).

Table 2 : MIC₅₀ (in μ M) and Synergistic Factor for mixtures of PGLa and magainin peptides. The average values and SD of MIC₅₀ as well as the synergistic factor (1/CI, in brackets) obtained from the experimental fitting (Figures 1 and 2) are shown for the peptides alone and in mixtures. Usually, data are from 2 - 8 experiments; when no SD is shown only one experiment has been analyzed.

			Magainin 2		
			amide	GGC_amide	GGs_amide
Average MIC ₅₀ +/-SD			14.48 $\pm$ 3.74	5.45 $\pm$ 0.76	14.11 $\pm$ 5.10
PGLa	L18W	6.55 $\pm$ 1.16	1.41 $\pm$ 0.46 (7.61 $\pm$ 2.12)	1.58 $\pm$ 0.07 (4.04 $\pm$ 0.84)	0.88 (11.02)
	L18W_GGC	2.80 $\pm$ 1.16	1.53 $\pm$ 0.59 (3.4 $\pm$ 0.84)	1.95 $\pm$ 0.73 (2.30 $\pm$ 0.59)	1.66 (2.30)
	L18W_GGS	10.40 $\pm$ 1.16	1.28 $\pm$ 0.56 (10.43 $\pm$ 3.98)	2.35 $\pm$ 0.06 (2.82 $\pm$ 0.07)	1.52 $\pm$ 0.00 (7.87 $\pm$ 2.53)
	L18W_GGA	6.04 $\pm$ 0.02	1.34 (5.60)	2.00 $\pm$ 0.52 (2.96 $\pm$ 0.57)	1.66 (4.68)

In equimolar combination with magainin, with magainin-GGC or with magainin-GGS (carboxy-amidated in all cases), all of PGLa, PGLa-GGC, PGLa-GGS and PGLa-GGA (L18W in all cases) exhibit MIC₅₀ in the 1-2 μ M range (MIC₁₀₀ of 1.6-3.1 μ M), which corresponds to synergistic enhancements between 2.3 and 11 (Table 2). Notably, a closely related MIC₁₀₀ of 1.3 μ M was published for the preformed covalently heterodimer magainin-GGC/PGLa-GGC [48], where the small difference can merely represent differences in conditions between laboratories and the day-to-day reproducibility between experiments. Interestingly, a mixture of magainin-GGS and PGLa-GGS is equally active although in this case the cysteines have been replaced by serines. The synergism of this mixture when compared to the individual magainin-GGS and PGLa-GGS derivatives is about eight-fold. When compared to PGLa-GGS, the PGLa-GGA derivative exhibits a similar behaviour when tested alone or in combination with magainins suggesting that neither a cysteine-mediated covalent bond nor a serine-driven hydrogen bonding interaction are required for the improved activities of the extended sequences.

When the MICs of the extended sequences are compared to each other, magainin 2 and PGLa carrying the GGC extension seem to be more efficient in antimicrobial assays than those carrying the GGA or GGS tri-peptide extensions. In some experiments the pH of the MH media turned out to range between 8 and 9, which are conditions favourable to cystine bridge formation. Indeed, mass spectra indicate that only in the presence of the cysteines some dimers co-exist with monomers. Therefore, we also tested antimicrobial activities at lower pH where the chemistry is less favourable to cystine-bridge and thereby dimer formation. When the pH was adjusted to 7.2 or to 5.5, the MICs of the peptides tended to decrease-when at the same time the MICs of the two GGC carrying peptides were hardly dependent on the pH at all. This observation suggests that at the neutral and lower pH the peptides become more readily available for antibacterial action, and/or that the bacteria are more sensitive to antimicrobial action. On the other hand, the formation of dimers seems to have a small or no effect on the MICs.

Table 3 : MIC₅₀ values (in μM) as a function of the pH of the Mueller Hinton medium.

The values at pH8.5* are the ones obtained without adjustment of the pH medium. The SD are typically $\pm 1 \mu\text{M}$ (cf. Table 2).

		pH 8.5*	pH 7.2	pH 5.5
Magainin 2	amide	14.48	9.39	2.10
	GGC_amide	5.45	5.04	4.58
	GGS_amide	14.11	8.69	4.49
PGLa	L18W	6.55	4.49	4.49
	L18W_GGC	2.8	4.59	2.03
	L18W_GGS	10.4	9.65	3.91
	L18W_GGA	6.04	6.03	4.55

Finally, a magainin analogue was synthesized where the deletion of valine-17 and insertion of an isoleucine at position 21 shifts the helical wheel arrangement between these residues (a helical wheel of this compound is shown in [58]). In addition F5 was replaced by tryptophan. This peptide by itself has a 4-fold increased antimicrobial activity (MIC₁₀₀ 3.1-6.3 μM) when compared to magainin 2. When mixed with PGLa or PGLa-GGC the antimicrobial activity remains unchanged in agreement with previous single-site mutations of magainin residues F16 and E19 which cause a reduced degree of synergism [41]. However, it should be noted, that the lowest MIC₁₀₀ obtained in the experiments presented in this paper is in the 1.6 μM range (Table S1). This minimal value observed in several mixtures seems independent of the MIC of the individual component as can be seen for example from the last column in Table 2 for magainin-GSS (in combination with different PGLa derivatives). As a consequence the synergistic enhancement tends to become smaller in combination with PGLa peptides that exhibit strong antimicrobial activities already as individual components.

Membrane topology of PGLa-GGX alone and in the presence of magainin 2-GGX peptides

In previous experiments the alignment of the PGLa helix has been found to be controlled by sensitive equilibria and to adopt an almost continuous range of alignments relative to the normal of PC membranes that have been prepared from saturated fatty acyl chains [45]. In particular in thin membranes the peptides exhibit an inherent tendency to adopt transmembrane tilt angles, an effect that is strongly enhanced in the presence of magainin 2 [44, 45]. Investigating the topology of PGLa peptides in di-C10:0-PC therefore provides a sensitive test for the peptides' tendency to adopt transmembrane alignments. Changes in membrane alignment can be directly monitored by ^{15}N solid-state NMR experiments of helical peptides reconstituted into uniaxially oriented bilayers, where a ^{15}N chemical shift in the 200 ppm range is indicative of a transmembrane orientation of helical domains, and values < 100 ppm correlate with alignments perpendicular to the membrane normal [59].

Previous investigations show that in di-C10:0-PC bilayers [^{15}N -Ala14]-PGLa (in the absence of magainin) exhibits a maximal intensity at around 125 ppm indicative of orientations close to the magic angle (ca. 55°), which changes to 170 ppm in the presence of magainin 2 [60]. This increase in chemical shift shows that PGLa adopts a more transmembrane tilt angle in the presence of magainin 2 [45]. Here we extended these investigations by studying the PGLa L18W variant that has been used previously in studies of antimicrobial action and synergism [41]. When labelled with ^{15}N at the alanine-14 position this mutant exhibits a chemical shift in di-C10:0-PC bilayers of 102 ± 10 ppm (Fig. 3A). Similar chemical shifts are observed when this sequence is extended by the GGC tripeptide (96 ppm) used in previous cross-linking studies [47] or the GGS extension (98 ppm) that we study here as an additional control (Table 2; Fig. 3B,C). Upon addition of magainin 2 the spectra of all three PGLa variants show two well defined, about equally intense resonances in the 200-210 ppm range and around 85-95 ppm (Fig. 3D-F). Thereby the spectra are indicative of a transmembrane and an in-planar population in slow exchange on the 10^{-4} sec time scale. In average the chemical shift increased by 25 to 50 ppm due to the presence of magainin 2 (Fig. 3) indicating that PGLa adopts a

more transmembrane topology. Upon addition of magainin 2-GGC the chemical shifts and topological distributions (Fig. 3 G-I) are similar than the ones obtained after addition of magainin 2 (Fig. 3D-F).

Figure 3. Proton-decoupled ^{15}N solid-state NMR spectra of [^{15}N -Ala14]-PGLa (A,D,G), [^{15}N -Ala14]-PGLa-GGC (B, E, H) and [^{15}N -Ala14]-PGLa-GGS (C,F,I) reconstituted into di-C10:0-PC bilayers oriented with the normal parallel to the magnetic field direction. Spectra were recorded in the absence (A-C) of other peptides, in the presence of unlabelled magainin 2-NH₂ (D-F) or magainin 2-GGC (G-I). The spectra were recorded at room temperature.

Notably, the synergism of the mixtures shown in Figures 3D-I varies between 2.3 and 10.4 (Table 2) when at the same time the amount of transmembrane peptide remains in the range of 50 ± 6 %. In order to compare the tendency of the PGLa peptides to adopt transmembrane alignments due to the presence of magainins a correlation graph was established including data from previous investigations (Fig. 4). There seems no correlation between the two experimental data.

Figure 4: Correlation between the TM alignment of PGLa in the presence of magainin and the synergistic activity (Table 2). The peptides were reconstituted into oriented di-C10:0-PC (Figure 3). The symbols represent: PGLa (circle), PGLa L18W (square), PGLa L18W-GGC (diamond), PGLa-L18W-GGS (pentagone), and NBD-PGLa (triangle). Open symbols represent mixtures with magainin 2-COOH (free carboxy terminus), full when mixed with magainin 2-NH₂, crossed when mixed with magainin 2-GGS and half-empty when mixed with rhodamine-magainine-COOH. The star represents the PGLa spectrum in the presence of magainin-COOH when investigated by solid-state NMR in in *E. coli* lipid extract (open star, Fig. 5D), in the presence of POPC (black/white star) or POPC/POPG (white/black star) [45]. The fluorophore-labelled peptides were investigated in [60]. The error in determining the transmembrane ratio is typically 10%.

Therefore, we also reconstituted [$^2\text{H}_3$ -Ala10, ^{15}N -Ala14]-PGLa in supported lipid bilayers made from *E. coli* lipid extract and investigated the topology of its helical domain by solid-state NMR. The resulting ^{15}N spectrum (Fig. 5A) shows a predominant peak at 87 ± 4 ppm from the labelled peptide bond as well as a sharp intensity at 31 ppm which is probably from the amine of PE lipids that are frequent in bacterial membranes but carry ^{15}N only at the low 0.4 % natural abundance of this isotope. When an equimolar mixture of [^{15}N -Ala14]-PGLa and unlabeled magainin 2 is investigated the chemical shift of 89 ± 4 ppm remains identical within experimental error (Fig. 5D). The deuterium solid-state NMR spectra of the $^2\text{H}_3$ -Ala10-site of PGLa exhibit a quadrupolar splitting of about 12 kHz in the presence or absence of magainin 2 as well as a central intensity of natural abundance water (HDO) associated with the oriented membranes. Finally the ^{31}P solid-state NMR spectra of the phospholipids show that despite the well-oriented ^{15}N spectra of the same sample considerable orientational dispersion of the phospholipid head groups with a chemical shift anisotropy of about 37 ppm. Although the main intensity corresponds to phospholipids that align with their long axis parallel to the sample normal (24 ppm) major intensities up to -13 ppm suggest additional topological distribution as one would observe from spherical (powder pattern) or cylindrical structures [61, 62].

Figure 5. Proton-decoupled ^{15}N (A,D), ^{31}P (C,F) and ^2H (B,E) spectra of [^{15}N -Ala14, $^2\text{H}_3$ -Ala10]-PGLa in *E.coli* lipid extract membrane oriented on glass plates in the absence (A-C) and in the presence of unlabeled magainin 2-COOH. The peptide concentrations are 2 mole % (panels A-C) and 1 mole % of each peptide in the peptide mixture (D-F), the spectra were recorded at 310K.

DISCUSSION

A covalent linkage does not improve synergism

The synergistic enhancement of antimicrobial activities has been observed in mixtures involving conventional antibiotics, detergents and peptides [40, 42, 51, 63-66]. Interestingly, the peptides investigated here are produced and stored as a cocktail already in their natural environment. Although synergistic effects could potentially be useful to treat bacterial infections including multiresistance little mechanistic data are available that provide explanations on a molecular level. In cellular environments synergistic effects can sometimes be explained by one compound helping the other to access its target [66]. However, here we investigated the synergism between two antimicrobial peptides of the magainin family that are an order of magnitude more active when calcein release activities from model membranes are investigated [41, 47, 48]. The observation that a preformed heterodimer exhibits increased calcein release activity from egg-PC

vesicles when compared to the individual peptides, albeit not in antimicrobial assays [48], seemed suggestive that close interactions between the peptides are of importance. Therefore direct interactions in the bilayer environment had been suggested initially [41, 44] however, these could so far not be confirmed in follow-up experiments [45, 49, 60]. Here, we investigate PGLa and magainin peptides with GGC extensions that have previously been designed for cross-linking studies in membranes [48]. Our experiments show that magainins with slightly modified tripeptide extensions, that are unable to form covalent cross links, exhibit less antimicrobial but even higher synergistic activities, demonstrating that the covalent bond is not a requirement for enhancement (Table 2).

Sequence alterations that affect synergism

When calcein release has been studied in the presence of mixtures of PGLa and magainin 2 the dye efflux is most pronounced when equimolar mixtures of the peptides were applied [41]. Interestingly the peptides have to fulfil specific requirements and synergism is only observed for some cocktails of antibiotics [63], detergent [64] or peptides [40, 65], as well as combinations of peptides with other compounds [42, 64, 66] but not for any combination of sequences. Here we show that synergisms remains active when the L18 position of PGLa is replaced by tryptophan (Table S1). In contrast the charge of the E19 site seems important as glutamine or lysine replacements affect the synergistic interaction with magainin 2 [41, 49]. Furthermore, it was possible to modify either the amino [60] or the carboxy terminus of the peptides (Tables 2 and 3) without abolishing synergistic interactions. Therefore, although the peptide interactions are somehow linked to the peptide sequences, they are not specific like receptor-ligand interactions. In contrary their presence has been difficult too proof in solid-state NMR [49] and fluorescence spectroscopy investigations [60], although the large size of the fluorophores used in the latter approach may influence the peptide-membrane and peptide-peptide interactions. Notably effects on synergism are obtained for both amino- (decrease; [60]) and carboxy-terminal modifications (increase; Table 2) suggesting that the peptide as a whole rather than a few specific interaction sites are involved.

How does the membrane topology of PGLa relate to synergism?

Because in phosphatidylcholine membranes made of saturated fatty acyl chains PGLa was shown to adopt transmembrane alignments in the presence of magainin 2 [44, 45] pore formation by heterologous transmembrane helical bundle has been suggested to explain synergistic enhancements [44]. However, in bilayers representing more closely the natural composition of biological membranes both peptides remain in a stable alignment parallel to the surface [45, 46]. In this context it should be noted that testing the alignment of ^{15}N -PGLa in di-C10:0-PC is quite sensitive to even small changes in the peptide sequence. For example, whereas the native PGLa exhibits a chemical shift of 125 ppm this value shifts to 100 ppm (Fig. 3A) for the L18W mutation, corresponding to a change in tilt angle of 12° or of the pitch angle by 150° , or a combination thereof. In contrast the ≤ 10 ppm difference observed upon extending with the tripeptide GGX (Fig. 3B,C) reflects a comparatively small effect at the alanine-14 site.

Upon addition of magainins, the broad peak intensities of PGLa (L18W) peptides, with or without the tripeptide extensions, split into two well-resolved peak intensities where the chemical shift values are indicative that about half the helices insert into the membrane and the remaining fraction a more surface-oriented alignment (Figs. 3D-I). In average a more inserted configuration is obtained for all of the peptide combinations investigated here (Fig. 3D-H). This contrasts the addition of NBD fluorophores to the amino-terminus of PGLa which resulted in a more in-plane alignment. Notably, the addition of NBD to PGLa as well as of magainins to the NBD-PGLa resulted in a broadened topological distribution as revealed by the ^{15}N intensities [60].

Although these measurements are of interest for our understanding what makes a peptide like PGLa respond to small modifications of the membrane or the presence of magainin 2 [45] there seems no correlation between these topological changes observed in di-saturated PC or PC/PG membranes [45, 46] and the synergistic enhancement of antibacterial activity (Figure 4). In contrary, solid-state NMR investigations on model membranes of more natural composition such as POPC, POPC/POPG [45, 46] or *E. coli* lipid extracts (Fig. 5) reveal that both peptides adopt stable alignments parallel to the membrane surface also when added in conjunction. Notably, the ^{15}N chemical shift of $[^{15}\text{N}\text{-Ala14}]\text{-PGLa}$ is within experimental error identical in all three membranes and

does not change significantly when magainin 2 is added at equimolar amounts (Fig. 5A,B,D,E) [45, 46]. In contrast, when ^2H solid-state NMR measurements of [$^2\text{H}_3$ -Ala10]-PGLa are compared from the *E.coli* lipid bilayers (Fig. 5B,E) or DMPC or DMPC/DMPG [44, 67] the different quadrupolar splittings (12 kHz and 30 kHz) reflect the different topologies in these bilayers, suggesting again that conclusions about the antimicrobial action and synergistic interactions of antimicrobial peptides should be considered with care when measured in saturated membranes.

Alternative models for synergistic interactions

Together, these data are suggestive that alternative models explaining synergistic interactions in membrane have to be found. Interestingly, the presence of 2 mole% PGLa causes considerable orientational heterogeneity at the level of the phospholipid head groups. The ^{31}P NMR spectra of supported *E.coli* lipid membranes at 310 K cover an anisotropy of about 37 ppm (Fig. 5C) which compares well to the PE and PG tensors under these conditions [68, 69]. Whereas the intensity at 24 ppm correlates with phospholipids with their long axis parallel to the sample normal, i.e. they are well-aligned relative to the surfaces of the glass support [70], the intensities at lower chemical shift reveal considerable orientational dispersion. This could arise from the conformational details at the level of the lipid head group, as observed upon changes in the electric surface charge [71], or from the lipid molecule as a whole. In the latter case major supramolecular rearrangements may be involved such as the loss of orientational order of the sample [72], the formation of cylindrical tubes of large diameter [61], membrane thinning and/or high local curvature [73]. Notably the intensities between 20 ppm and -15 ppm considerably increase when 1 mole% each of PGLa and magainin 2 have been reconstituted (Fig. 5 F) and it is tempting to speculate that the synergism that comes with such mixtures relates to such changes in the lipid macroscopic arrangements and phase properties [45].

A view emerges in which synergism occurs for example by lipid-mediated rather than strong direct interactions between the peptides. Here we used and refined sequences that have been developed early to cross-link PGLa and magainin peptides [47]. These studies indicated that the peptides preferentially cross-link when carrying cysteines that favour a relative alignment of the peptides parallel to

each other. The unsaturated fatty acyl composition of the egg yolk PC / egg yolk PG membranes used in these studies is suggestive of an alignment of the peptides parallel to the bilayer surface [45, 47]. The high peptide density that usually occurs during these experiments is suited to reveal relative interaction preferences rather than indications of absolute interaction strength (e.g. [74]). Furthermore the positive charge of the amino-terminus of PGLa-GGC and magainin-GCC prepared for the cross-linking investigations was neutralized by acetylation [48, 75], which probably improves the interactions between these globally cationic peptides. Accordingly, RET [60], ITC (A. Marquette & B. Bechinger, unpublished) and solid-state NMR measurements [49] agree that the interaction between the two sequences are weak.

Such interactions could for example be electrostatic and/or mediated by the lipids [45, 60]. The important role of the magainin 2 E19 site suggests that electrostatic interactions indeed play an important role [41]. Although overall cationic the charge distribution along the peptide structures is asymmetric and therefore a dipolar moment is created when the peptide interacts with the membrane in its helical conformation [60] favouring certain relative helix orientations over others [75, 76]. Notably, we have recently observed mesophase-like arrangements with head-to-head/tail-to-tail molecular dipoles of an in-plane oriented cationic amphipathic peptide by fluorescence quenching spectroscopy [77] and similar interactions could occur in magainin / PGLa mixtures.

In summary, the results presented in this paper do not reveal a correlation between the peptides' tendency to adopt transmembrane alignments. In contrast experiments with *E.coli* lipid extracts suggest that synergy occurs when both PGLa and magainin remain aligned parallel to the membrane surface. Furthermore, the possibility to form covalent heterodimers does not improve the capacity of PGLa / magainin mixtures to interact in a synergistic manner. Therefore, we suggest that more indirect interactions, possibly through the lipid phase are responsible and that modulations of the lipid packing may play an important role for synergistic interactions.

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SUPPLEMENTARY INFORMATION

Investigations of the synergistic enhancement of antimicrobial activity in mixtures of magainin 2 and PGLa

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Table S1: The antimicrobial activities (MIC₁₀₀ in μM) of magainin 2 and PGLa, derivatives and combinations thereof.

			Magainin 2			
			COOH	amide	GGC_amide	GGs_amide
MIC ₁₀₀			25-12.5	25-12.5	12.5-6.3	25-12.5
PGLa		12.5	6.3-3.1	3.1	3.1	
	L18W	12.5-6.3	3.1-0.8	3.1-1.6	3.1-1.6	1.6
	L18W_GGC	6.3-3.1	6.3-3.1	3.1-1.6	3.1-1.6	1.6
	GGC	6.3	6.3-3.1	3.1	3.1	
	L18W_GGS	12.5	3.1	3.1-1.6	3.1	3.1-1.6
	L18W_GGA	25-6.3		1.6	3.1	3.1-1.6

Research Highlights

PGLa orients parallel to the surface of membranes made from *E. coli* lipids in the absence or presence of magainin 2

Magainin strongly disorders the phospholipid head groups of *E. coli* lipids

Synergism does not require pronounced direct/covalent peptide-peptide interactions

Lack of correlation between the tendency of PGLa variants to span thin membranes and their synergistic activity.