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# Influence of C-terminal amidation on the antimicrobial and hemolytic activities of cationic α-helical peptides*

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*Abstract*: The effect of C-terminal amidation on the antimicrobial and hemolytic activities of antimicrobial peptides was studied using three cationic peptides which form amphiphilic α-helices when bound to membranes. The natural antimicrobial peptide PGLa, the designer-made antibiotic MSI-103, and the cell-penetrating "model amphipathic peptide" (MAP) are all amidated in their original forms, and their biological activities were compared with the same sequences carrying a free C-terminus. It was found that, in general, a free COOH-terminus reduces both the antimicrobial activity and the hemolytic side effects of the peptides. The only exception was observed for MSI-103, whose antimicrobial activity was not decreased in the acid form. Having shown that the therapeutic index (TI) of this novel peptide is significantly higher than for the other tested peptides, with high antibiotic activity and little undesired effects, we suggest that it could be a useful starting point for further development of new peptide antibiotics.

*Keywords*: antimicrobial peptides; cationic α-helices; amphipathic peptides; C-terminal modifications; designed peptide antibiotics; biological assays.

## INTRODUCTION

Antibacterial peptides are found in many organisms and form an important first-line defence against microbes [1–4]. The importance of such peptides is exemplified by a human disease in which the lack of the antimicrobial peptide LL-37 leads to severe symptoms and is often lethal [3]. Many of these peptides are cationic and form amphiphilic α-helices when bound to lipid bilayers, hence they kill bacteria presumably by disrupting their membranes. They operate in a receptor-independent mode, as the enantiomeric *all-D* analogs exhibit the same activities as the original *all-L* wild-type peptides [5]. Bacteria are not expected to develop immunity against these naturally occurring antibiotics, by which the cellular integrity is destroyed in a matter of minutes [3,4]. The peptides are, therefore, promising candidates as new drugs against multiresistant bacteria, and some have already been used in clinical trials [1,4]. The only fundamental drawback of these agents is their more or less pronounced ability to dis-

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rupt also the cellular membranes of the eukaryotic organism to which they are being applied. In this regard, hemolytic side effects need to be minimized in order to turn a comparatively nonspecific antimicrobial peptide into a universally useful drug. There is high expectation of success, since the cell surface differs significantly between prokaryotes and eukaryotes not only with regard to lipid composition and cholesterol content, but also with regard to charge, the transmembrane potential, and other factors.

In the search for improved antibiotics, several groups have modified naturally occurring antimicrobial peptides to make them more active and more specific. It would be highly useful to identify the physicochemical parameters which provide a peptide with a high activity against microbes, and at the same time a minimal activity against the cells of the host organism. To test a compound quantitatively, antimicrobial assays are used to determine the minimum inhibitory concentration (MIC) of the peptide against different bacterial strains, and hemolysis assays are used to measure the percentage of hemolysis induced in fresh erythrocytes. A low MIC and low hemolytic effect are the desired criteria for a promising drug. To allow for comparison of different peptides in a quantitative manner, a therapeutic index (TI) may be defined as the ratio of MIC over a certain hemolytic threshold concentration.

To improve the TI of naturally occurring antimicrobial peptides and designer-made analogs, many studies have been performed in which properties such as charge, length, hydrophobic moment, and amphiphilicity have been systematically varied by selective mutations of the sequence (see [6,7] and refs. therein). In most of these studies, the peptides used were amidated at the C-terminus (e.g., see [6–9]), being the form in which many but not all natural antimicrobial peptides occur [10]. For example, of the two well-known antimicrobial peptides found in the skin of the African frog *Xenopus laevis*, PGLa is amidated at the C-terminus while magainin carries a free acid. In general, amidated peptides have been observed to exhibit higher antimicrobial activity, but they are also more hemolytic, hence it is not self-evident whether amidation improves the TI or not [8,11].

In the present study, three cationic amphipathic α-helical antimicrobial peptides have been studied with amidated and acidic C-termini (see Table 1 for sequences). Specifically, we have compared the naturally occurring peptide PGLa from *X. laevis* [12] with MSI-103, which is a designer-optimized 21-mer sequence based on PGLa as a template. The main design concept was to replace Gly-1 and Ala-8 with Lys to increase both the positive charge and the amphipaticity of the peptide, factors shown to be important for activity. The resulting peptide was then simplified into a repeated heptameric sequence, which has higher antimicrobial and hemolytic activities than PGLa [10,13]. We also included the "model amphipathic peptide" (MAP) in this study, which is an 18-mer designer-made cell-penetrating peptide [14]. Since MAP has a similar sequence and charge as the other peptides it can be assumed to have a comparable antimicrobial activity. All three peptides have characteristic amphiphilic helical wheel projections (Fig. 1), with one charged face carrying several Lys residues, an opposite face carrying only Ala residues, while on the flanking faces, the more hydrophobic Ile, Leu, and Val are found. In PGLa and MSI-103 also two or three Gly residues are clustered on one side of the helical wheel. In the original design, all three peptides are amidated, but in this study we have also synthesized the analogs with free acidic C-termini. The three peptides are currently being studied by solid-state NMR in our group [15–18], hence we will try to relate the knowledge gained from these structural studies to the results from the biological tests presented here.

**Table 1** Peptide sequences.

| Name | Sequence | Molecular mass [Da][a] | Total charge[b] |
|---|---|---|---|
| PGLa-$NH_2$ | GMASKAGAIAGKIAKVALKAL-$NH_2$ | 1968.5 | +5 |
| PGLa-COOH | GMASKAGAIAGKIAKVALKAL-COOH | 1969.5 | +4 |
| MSI-103-$NH_2$ | KIAGKIAKIAGKIAKIAGKIA-$NH_2$ | 2062.7 | +7 |
| MSI-103-COOH | KIAGKIAKIAGKIAKIAGKIA-COOH | 2063.7 | +6 |
| MAP-$NH_2$ | KLALKLALKALKAALKLA-$NH_2$ | 1875.5 | +6 |
| MAP-COOH | KLALKLALKALKAALKLA-COOH | 1876.5 | +5 |

[a]Value from mass spectrometry (MS); in all cases, the expected theoretical mass was found.
[b]Calculated from charged lysine residues and terminal groups.

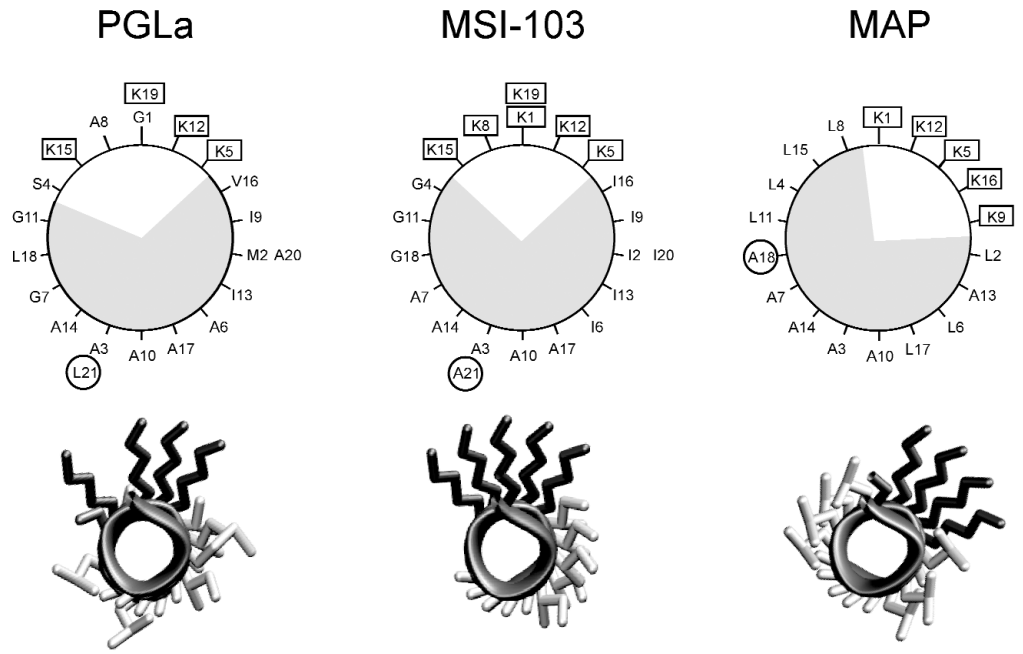


**Fig. 1** Helical wheel projections of the amphiphilic α-helical peptides PGLa, MSI-103, and MAP. Charged residues are marked by rectangles, and the C-terminal amino acid by a circle. The hydrophobic sector is shaded. In the panels below, an end-view of the helix is shown for each peptide with amino acids in a stick representation.

## RESULTS

All the original peptides, PGLa, MSI-103, and MAP, carry an amidated C-terminus. In order to distinguish the amidated forms from those carrying a free acid at the C-terminus, they are labelled –$NH_2$ or –COOH, respectively, in the text. (No suffix is added when both forms of a peptide are being discussed.) The antimicrobial activities of all six peptides were measured using an antibacterial assay on three Gram-negative and four Gram-positive bacterial strains. The MIC values were determined as the lowest concentration where no bacterial growth was observed. Using a two-fold dilution series, it is not possible to state exact values of MIC, and a slight change in activity can give rise to a factor of two in the apparent MIC value. Thus, for a significant distinction, the MICs should differ by at least a factor of four. The highest peptide concentration used in these experiments was 128 μg/ml, and when this concentration did not inhibit growth the peptide was considered to be inactive against that bacterial strain. The antimicrobial results are presented in Fig. 2 and summarized in Table 2.

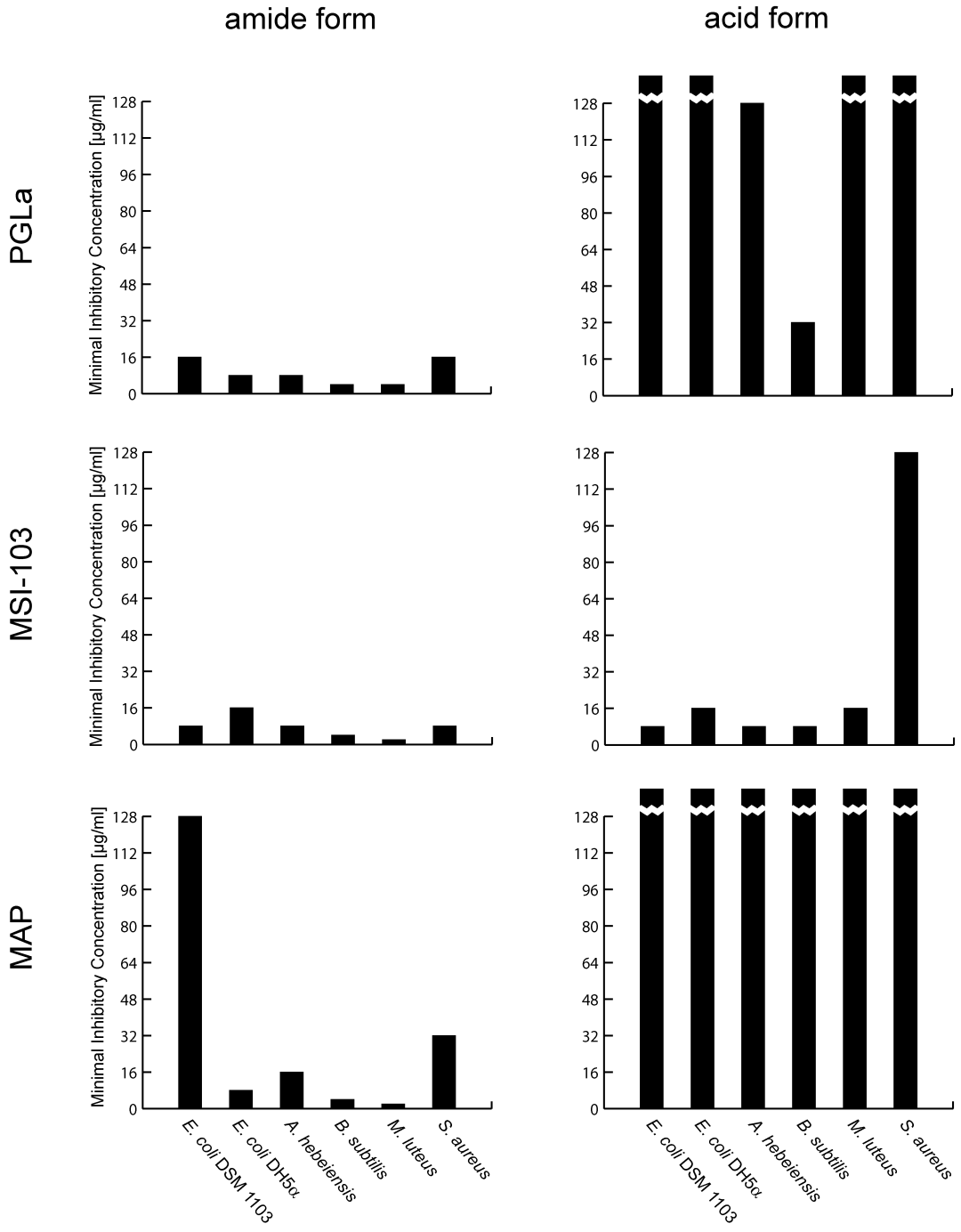


**Fig. 2** Illustration of the antimicrobial activities of PGLa, MSI-103, and MAP. For each peptide, the MIC values are represented as bars over each of the six bacterial strains against which the amidated peptides showed activity. The panels on the left show the results for the original peptides with an amidated C-terminus, and the right-hand panels correspond to peptides with a free C-terminus. A high MIC value implies low antimicrobial activity. When the MIC was found to be higher than 128 µg/ml, this is indicated by a broken column. Due to the dilution series used, the margin of error in MIC values is a factor of two. (Data on *K. rhizophila* are not illustrated here, since all peptides were inactive except for MSI-103-$NH_2$ showing a slight effect.)

**Table 2** Antimicrobial activity of the peptides.

| | Minimal inhibitory concentration (MIC) [μg/ml] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PGLa-$NH_2$[a] | PGLa-COOH | MSI-103-$NH_2$[a] | MSI-103-COOH | *MSI-103-$NH_2$*[b] | *MSI-103-COOH*[b] | MAP-$NH_2$ | MAP-COOH |
| **Gram-negative** | | | | | | | | |
| *E. coli* (DSM 1103) | 16 | >128 | 8 | 8 | – | – | 128 | >128 |
| *E. coli* (DH 5α) | 8 | >128 | 16 | 16 | *4* | *8* | 8 | >128 |
| *A. hebeiensis* (DSM 586) | 8 | 128 | 8 | 8 | *8* | *8* | 16 | >128 |
| **Gram-positive** | | | | | | | | |
| *B. subtilis* (ATCC 6633) | 4 | 32 | 4 | 8 | *8* | *16* | 4 | >128 |
| *M. luteus* (DSM 1790) | 4 | >128 | 2 | 16 | *8* | *4* | 2 | >128 |
| *S. aureus* (DSM 1104) | 16 | >128 | 8 | 128 | – | – | 32 | >128 |
| *K. rhizophila* (DSM 348) | >128 | >128 | 64 | >128 | – | – | >128 | >128 |

[a]In previous studies [10,13] the MIC of MSI-103-$NH_2$ was similar to in this work, while the MIC of PGLa appeared to be higher than found here, possibly due to different bacterial strains used.
[b]Second series of measurements, which cannot be directly compared with the other data.

Overall, MSI-103-$NH_2$ is seen to be the most active peptide with the lowest MICs against all bacterial strains, followed by PGLa-$NH_2$ and MAP-$NH_2$. MSI-103-$NH_2$ is the only peptide that had any significant activity against *K. rhizophila*. Interestingly, MAP-$NH_2$, which is a synthetic peptide designed to be cell-penetrating, showed almost as much antibacterial activity as the natural antimicrobial peptide PGLa-$NH_2$ against five of the strains, indicating that this activity is due to general physicochemical interactions between cationic amphiphilic peptides and bacterial membranes.

When the amidated peptides are compared with the respective analogs carrying a free C-terminus, remarkable differences are found for the three underlying sequences. MAP-COOH completely lost all antibacterial activity compared to MAP-$NH_2$ against all strains tested. PGLa-COOH lost the original activity of its PGLa-$NH_2$ parent against five of the strains, showed a very low activity against *A. hebeiensis*, and a weak activity against *B. subtilis*, which was still much lower than for PGLa-$NH_2$. In contrast to the situation observed for MAP and PGLa, the peptide MSI-103-COOH showed the same activity as MSI-103-$NH_2$ against all gram-negative strains and *B. subtilis*, and it maintained a somewhat reduced but still significant activity against *M. luteus* and *S. aureus*. It thus appears that MSI-103-COOH is a potent antibiotic, unlike the inactive acid forms of MAP and PGLa.

In hemolysis assays, the ability of the peptides to release hemoglobin from human erythrocytes was measured at different peptide concentrations. Here, 0 % hemolysis was determined by a test without any peptide, and 100 % was induced by adding the detergent Triton-X to the erythrocytes. For each peptide concentration the percentage hemolysis was measured and is stated as a number. Hemolysis curves for the peptides are shown in Fig. 3, and the percentages of hemolysis at some selected peptide concentrations are listed in Table 3. It is seen that MAP has a very strong hemolytic activity compared to PGLa and MSI-103. At concentrations close to the MIC values for most bacterial strains, both MAP-$NH_2$ and MAP-COOH cause nearly 100 % hemolysis. Therefore, even if the amidated form of this peptide has a pronounced antibacterial activity, it is clearly useless as an antimicrobial drug. PGLa-$NH_2$ and MSI-103-$NH_2$, on the other hand, have reasonably low hemolytic activities, with similar curves for both peptides. These peptides show little hemolytic side effects at concentrations needed to kill bacteria, hence they are useful as antimicrobial drugs.

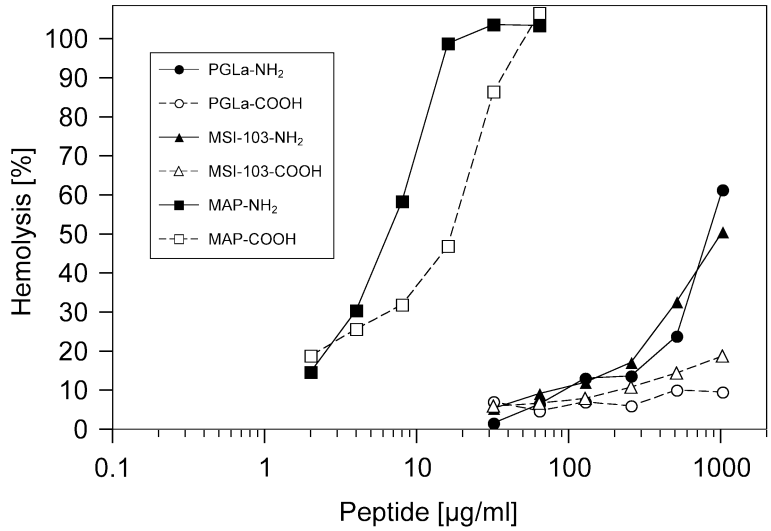


**Fig. 3** Diagram of the hemolytic activities of PGLa, MSI-103, and MAP. Filled symbols and continuous lines represent peptides with an amidated C-terminus, while open symbols and dashed lines are used for peptides with a free C-terminus.

**Table 3** Hemolytic activity of the peptides at selected concentrations.

| Peptide | Hemolytic activity in % at peptide concentrations [µg/ml] | | | |
|---|---|---|---|---|
| | 32 | 128 | 512 | 1024 |
| PGLa-$NH_2$ | 2 | 13 | 24 | 61 |
| PGLa-COOH | 7 | 7 | 10 | 10 |
| MSI-103-$NH_2$ | 5 | 12 | 33 | 51 |
| MSI-103-COOH | 6 | 8 | 14 | 19 |
| *MSI-103-$NH_2$*[a] | *N/A* | *9* | *45* | *76* |
| *MSI-103-COOH*[a] | *N/A* | *3* | *2* | *4* |
| MAP-$NH_2$ | 100 | 100[b] | 100[b] | 100[b] |
| MAP-COOH | 86 | 100[b] | 100[b] | 100[b] |

[a]Second series of measurements, which cannot be directly compared with the other data.
[b]Concentrations not measured, but 100 % hemolysis was observed already at 64 µg/ml.

Figure 3 shows that for all the peptides, the hemolytic activity is lower for the acid form (open symbols) than for the amide form (filled symbols). Removal of the C-terminal amide group does not, however, necessarily improve the use of a peptide as an antimicrobial drug. Namely, PGLa-COOH is less hemolytic than PGLa-$NH_2$, but the antibacterial activity is also much lower for the amidated form than for the acid form (see Fig. 2, Table 2). Hence, the selectivity of PGLa against prokaryotic and eukaryotic cells cannot be fundamentally improved by such C-terminal modification. The same arguments also hold for MAP. On the other hand, the hemolytic activity of MSI-103-COOH is much lower than for MSI-103-$NH_2$, while the antibacterial activities of the two forms are essentially the same. This remarkable observation suggests that specifically for MSI-103 a free C-terminus significantly improves its therapeutic potential.

To compare the peptides more quantitatively, a TI was calculated, which judges the relative activities of the peptides against bacteria and erythrocytes. Details about this calculation are given in the experimental section. A high TI means that the peptide has a strong activity against bacteria, and at the same time shows only little undesirable hemolytic effects. Due to the uncertainties in MIC and hemolysis values, TI is not an exact value, and differences of a factor two or less might not be significant. For each peptide interacting with each bacterial strain a separate index was evaluated, and the numbers are summarized in Table 4. Starting with the cell-penetrating sequence of MAP as the worst case, it is seen that the amidated peptide and the acid analog both have very low TIs because of their strong hemolytic activities. The naturally occurring peptide PGLa-$NH_2$ has better TIs against both Gram-negative and Gram-positive bacteria, while PGLa-COOH has somewhat lower values due to its reduced antibacterial activity, which is not fully compensated by the lower hemolytic activity. Compared to PGLa-$NH_2$, the original designer-made peptide MSI-103-$NH_2$ has similar TIs against Gram-negative bacteria, and higher TIs against gram-positive bacteria. Finally, MSI-103-COOH has the highest TIs against all gram-negative bacteria and against *B. subtilis*. The TIs of MSI-103-COOH are exceeded by MSI-103-$NH_2$ and PGLa-$NH_2$ only against *M. luteus* and *S. aureus*. It is worth noting that amongst the six bacterial strains tested, MSI-103-COOH is the best antibiotic against four strains and MSI-103-$NH_2$ against the other two. This observation suggests that MSI-103 is a very active peptide and highly promising candidate for designing peptides with even better TI.

**Table 4** Therapeutic index. The best peptide against each bacterial strain is highlighted in bold.

| | Therapeutic index | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PGLa-$NH_2$ | PGLa-COOH | MSI-103-$NH_2$ | MSI-103-COOH | *MSI-103-$NH_2$*[a] | *MSI-103-COOH*[a] | MAP-$NH_2$ | MAP-COOH |
| **Gram-negative** | | | | | | | | |
| *E. coli* (DSM 1103) | 6.2 | 2.0 | 11 | **27** | – | – | 0.010 | 0.004 |
| *E. coli* (DH5α) | 12 | 2.0 | 5.3 | **13** | *31* | ***250*** | 0.17 | 0.004 |
| *A. hebeiensis* (DSM 586) | 12 | 4.0 | 11 | **27** | *16* | ***250*** | 0.083 | 0.004 |
| **Gram-positive** | | | | | | | | |
| *B. subtilis* (ATCC 6633) | 24 | 16 | 21 | **27** | *16* | ***125*** | 0.33 | 0.004 |
| *M. luteus* (DSM 1790) | 24 | 2.0 | **43** | 13 | *16* | ***500*** | 0.67 | 0.004 |
| *S. aureus* (DSM 1104) | 6.2 | 2.0 | **11** | 1.7 | – | – | 0.042 | 0.004 |

[a]Second series of measurements, which cannot be directly compared with the other data.

In order to ensure that the positive result for MSI-103-COOH was not an artefact, a second series of experiments were independently performed with MSI-103-$NH_2$ and MSI-103-COOH. The unexpectedly high antimicrobial activity of the acid form especially had to be verified, as this activity had not been maintained for the acid forms of the other two peptides PGLa and MAP. In the second series of experiments, only four bacterial strains were examined and a limited range of peptide concentrations was employed for the hemolysis assays. The results are included in Tables 2 and 3 (not displayed in Figs. 2 and 3). The new antimicrobial data confirms our previous data on MSI-103-$NH_2$ and MSI-103-COOH, as the MIC values for the two peptides differ by no more than a factor of two. This finding confirms that the acid form of MSI-103 is as active against bacteria as the originally designed amidated form. The hemolysis assays also confirm the picture discussed above for all peptides, namely, that the acid form is less active than the amidated form. In fact, in the second series of experiments, the relative difference between MSI-103-$NH_2$ and MSI-103-COOH appears to be even more pronounced than in the first series, as this time the acid form of MSI-103-COOH had virtually no hemolytic activity at all, even at the highest peptide concentration tested. Due to this extremely low activity, the TIs calculated for the second set of data are much higher than the numbers obtained before, accentuating that MSI-

103-COOH is indeed an excellent candidate for a highly selective antimicrobial drug (see Table 4). Concerning the absolute values stated in the tables, it is obvious that they cannot be compared directly between different series of experiments carried out on different days by different individuals. A different batch of fresh blood was used for the second set of hemolysis assays, while, of course, in the first series, all six peptides were tested simultaneously using another batch.

## DISCUSSION

Many studies have revealed that the antimicrobial activity is related to the positive charge of peptides, with a higher activity for peptides with higher charge [7,9,19]. This was also one of the concepts used in the design of MSI-103 [10,13]. In the present study, the different peptides have a net charge of +4 to +7, calculated from number of lysines and charged terminal groups (see Table 1). Amidated peptides thus have a higher positive charge than those with a free C-terminus, and this may be part of the explanation as to why they are more active. However, it is clear that charge alone is not sufficient to explain the different activities, since MAP-COOH has the same charge as PGLa-$NH_2$ but a much lower activity. The activities of PGLa-$NH_2$ and MSI-103-$NH_2$ are comparable, even though PGLa has a two units lower charge. Part of the explanation as to why MSI-103-COOH is more active than PGLa-COOH and MAP-COOH may be the higher charge, but other factors also have to be involved.

One factor that has been identified as important for hemolytic activity is the helix stability [7,9,20]. In this respect, the two Gly residues of PGLa and three Gly of MSI-103 can be expected to reduce the helical propensity of these peptides compared to MAP, which may partly explain their lower hemolytic activities. This factor does not, however, explain the observed difference between peptides with different C-terminal groups. The hemolytic activity is also known to be correlated with the hydrophobicity of the peptide, with more hemolysis being induced by the more hydrophobic peptides [21]. Of the three peptides tested here, MAP is the most hydrophobic with eight Leu side chains on both sides of the charged face (see Fig. 1). MSI-103 carries six Ile, all on the same side of the helix, while PGLa carries two Ile, two Leu, and a Val, distributed over the hydrophobic face, hence the total hydrophobicity of these two peptides is similar. When a peptide has a free C-terminus, the net charge goes down compared to the amidated form, but the number of charged groups increases. Therefore, since the mean hydrophobicity is higher for amidated peptides they are expected to induce more hemolysis than the acid forms, which is indeed observed here. Comparing the three different types of peptide sequence with one another, the hemolytic effects correlate with the hydrophobicity. Since the activity of MAP is so much higher than of PGLa and MSI-103, other factors are likely also involved. One such factor could be the very different distribution of hydrophobic side chains along the helix (see Fig. 1).

It is known from previous solid-state $^{19}F$ and $^{2}H$ NMR studies that the amphiphilic helix of PGLa-$NH_2$ is aligned flat on the membrane surface at low peptide concentration, with its charged residues pointing toward the water phase [16,22]. At higher concentration, a tilted state has been found, where the helical axis gets inclined by about 35º with respect to the membrane plane, with the C-terminus pointing lower than the N-terminus [15,16]. This re-alignment has been explained by the formation of dimers, in which the two peptide helices are packed against one another with a stable crossing angle. The tilt angle of these homodimers is still too shallow to span the membrane, hence, if a pore is needed to destroy the bacterial membranes, it appears to be short-lived and not stable on the NMR timescale. Interestingly, when PGLa-$NH_2$ is mixed with magainin 2 (which has a free C-terminus) in a 1:1 ratio, it is found to be aligned almost upright in the membrane, which is compatible with the formation of a transmembrane pore [17]. Upon increasing the PGLa concentration even further, the peptides have been found to form extended aggregates that are immobile. Also for MSI-103-$NH_2$, we have recently found a flat surface-bound state at low concentration, and a tilted state at high concentration [18]. Therefore, this designer-made peptide is expected to form dimers in the same way as the naturally occurring PGLa-$NH_2$ and may thus have a similar mechanism of action. However, in the case of MSI-103, a lower concentration of peptide is sufficient to induce dimerization and aggregation com-

pared to PGLa-$NH_2$. In contrast, MAP-$NH_2$ was found to form large aggregates already at very low concentration and may have a rather different mode of action (Wadhwani, Bürck, Strandberg, Ulrich, unpublished results). If we may speculate here in view of the currently emerging structural results, it appears that dimerization is involved in antimicrobial activity, while aggregation might play a role in hemolysis. These ideas may explain the much higher hemolytic activity of MAP-$NH_2$, and it is plausible that aggregation is related to the hydrophobic side chains that are laterally exposed on the sides of the peptide. So far, no NMR studies have been performed on the corresponding peptides with free C-termini, but it is likely that the differences between the amidated and acid forms can be explained once detailed information on their alignment and aggregation in membranes is available.

## CONCLUDING REMARKS

The new peptide MSI-103-COOH, which has a high antimicrobial activity and low hemolytic side effects, has been identified as an improved antibiotic drug candidate compared to the original amidated form of the designer-made peptide MSI-103-$NH_2$. The two forms have similar antimicrobial activities, but removal of the C-terminal amide group reduces the hemolytic side effects, hence the TI of MSI-103-COOH is higher. In contrast to these findings with MSI-103, the two related amphiphilic α-helical peptides PGLa and MAP respond differently to a removal of their C-terminal amide, as also their antimicrobial activity is reduced when the C-terminus is unprotected. It therefore seems that a free acid at the C-terminus does not generally improve the TI of an antimicrobial peptide, and it may also enhance the sensitivity toward proteases. In the case of MSI-103, however, the acid form is a promising candidate for further optimization of the TI. By combining the current biological activity data with ongoing structural NMR studies on the alignment and self-assembly of such peptides in membranes, a better understanding of the relationship between structure and activity will be gained.

## EXPERIMENTAL

### Peptide synthesis

Peptide synthesis reagents and Fmoc-protected amino acids were purchased from Merck Biosciences (Darmstadt, Germany) and Iris Biotech (Marktretwitz, Germany). Solvents were purchased from Merck (Darmstadt, Germany). The peptides were synthesized on an Applied Biosystems 433A instrument, using standard solid-phase Fmoc protocols [23]. The crude material was purified by high-pressure liquid chromatography (HPLC) on a Vydac C18 column using an acetonitrile/water gradient. The identity of the products was confirmed by mass spectrometry (matrix-assisted laser desorption ionization with time-of-flight detection, MALDI-TOF). Analytical HPLC showed them to be at least 95 % pure.

### Antimicrobial activity

Antimicrobial activity was studied by a standard MIC assay, carried out with Gram-positive *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (DSM 1790), *Staphylococcus aureus* (DSM 1104), and *Kocuria rhizophila* (DSM 348), and with Gram-negative *Escherichia coli* (DSM 1103 and DH5α), and *Acinetobacter hebeiensis* (DSM 586). Bacteria were grown in Luria-Bertani medium at 37 °C and 230 rpm overnight, and diluted in 1 % trypticase soy broth. Microtiter plates (96 wells of 100 µl) were filled with 50 µl of 1 % TSB, and serial 2-fold dilutions of peptides were arranged in quadruple. The two final rows of each plate remained without peptide, so that the penultimate data point served as the positive control (no peptide) and the final one as the negative control (not inoculated). 50 µl of bacterial suspension was added to the wells (except for the final row of each plate) to give a final concentration of $10^6$ colony-forming units per milliliter. The plates were incubated at 37 °C for 20 h, and the

MIC was determined visually on the basis of turbidity as the lowest concentration inhibiting bacterial growth.

### Hemolysis assay

Hemolytic activity was examined by a serial 2-fold dilution assay, modified from previously published assays [24]. Citrate phosphate dextrose-stabilized blood bags with erythrocyte suspensions of healthy donors were obtained from the blood bank of the municipal hospital (Karlsruhe, Germany). Erythrocytes were washed twice with 9-fold excess of Tris buffer (172 mM, pH 7.6 at 0 °C) followed by centrifugation at 600 g for 10 min at 4 °C, and kept on ice in between. After the second wash, the erythrocytes were transferred from the sediment to a fresh tube with the same precooled buffer to be diluted to about 10 % (v/v) hematocrit, giving the stock cell suspension, which was kept on ice. For each peptide, serial 2-fold dilutions in Tris buffer (pH 7.6 at 37 °C)/dimethylsulfoxide (9:1 v/v) were prepared to have twice the desired end concentration (an equal volume of erythrocyte suspension will be added later to start the incubation). The stock cell suspension was further diluted to about 0.5 % (v/v). After preincubating for 3 min, 200 µl of the resulting erythrocyte dilution was transferred to each tube of the corresponding peptide serial dilution, to a final concentration of 0.25 % (v/v). For each dilution series, zero hemolysis was obtained by adding the erythrocytes to Tris buffer (pH 7.6 at 37 °C)/dimethylsulfoxide (9:1 v/v) and measuring the background lysis in the absence of peptide. For 100 % hemolysis, the erythrocytes were added to 0.2 % of Triton X-100 (Sigma, Germany) in the same buffer, giving a final concentration of 0.1 % Triton X-100. Incubation was performed at 37 °C for 20 min with gentle shaking. The tubes were centrifuged at 20 000 g for 5 min to pellet the cells, and the absorbance at 540 nm was recorded against water. The percentage lysis was then calculated relative to 0 % lysis with buffer and 100 % lysis by Triton X-100. The absorbance measurement was repeated three times, and the averaged values are used. Due to the very different activities of the peptides, MAP was measured in a lower concentration range than PGLa and MSI-103.

### Therapeutic index

To compare the relative acitivies of the peptides against bacteria and erythrocytes, a TI was defined and calculated as TI = {concentration required for 10 % hemolysis}/MIC. The hemolytic activity curve was used to interpolate the minimum peptide concentration giving 10 % hemolysis. In the second series of measurements on MSI-103-COOH only 4 % hemolysis was observed even at the highest peptide concentration, hence a value of 2000 µl /ml was used in this case. The MIC values were obtained for each bacterial strain (excluding *K. rhizophila* against which peptides were not active), and a value of 256 µl/ml was used as a lower estimate if a peptide was inactive. The TI was then evaluated for each bacterial strain by dividing the 10 % hemolysis concentration by the MIC values. A higher TI indicates that the peptide is more active against bacteria relative to its undesirable ability to destroy erythrocytes.

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