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Gramicidin S is active against both gram-positive and gram-negative bacteria

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Four linear and four cyclic analogs of gramicidin S (GS) in which D-Phe was replaced with either D-His, D-Ser, D-Tyr or D-Asn have been prepared by solid-phase peptide synthesis and characterized with respect to antibacterial, antifungal and hemolytic activity. Unlike previous reports, GS and a number of cyclic analogs were found to be active against gram-positive as well as gram-negative bacteria. GS showed MICs ranging from 3 to 12.5 μg/mL for gram-negative bacteria, compared to MICs of 3 μg/mL for gram-positive bacteria. Furthermore, these analogs were also found to exhibit antifungal activity. Unlike the cyclic analogs, all linear analogs were found to be inactive against a wide range of microorganisms tested, and showed low levels of hemolytic activity. The antibacterial activity was found to be highly dependent on the type of assay used, with solution-based assays showing greater activity against gram-negative bacteria than agar-based assays. The GS cyclic analogs were all less toxic than GS itself, with the analog containing the D-Phe to D-Tyr substitution showing the greatest activity of the synthetic analogs. Hemolytic activity in solution against human and sheep red blood cells paralleled antibiotic activity, with those peptides exhibiting greater antibiotic activity generally showing greater hemolytic activity. Membrane destabilization as monitored using the hydrophobic probe *N*-phenyl-1-naphthylamine was also found to parallel antibacterial and hemolytic activity of cyclic and linear analogs. These results indicate that GS and certain related analogs may have applications as broad-spectrum antibiotics and should be reevaluated for such purposes. © Munksgaard 1996.

Key Words: analogs; antibiotic activity; gramicidin S; hemolytic activity; gram-negative bacteria

Gramicidin S (GS) is a naturally occurring cyclic peptide antibiotic first isolated from *Bacillus brevis* by Gause and Brazhnikova (1). It has the sequence (Val-Orn-Leu-D-Phe-Pro)$_2$ and has been shown to possess a two-stranded β-sheet structure with the strands fixed in place by two type II′ β-turns made up by D-Phe and Pro residues (2–5). During the 1970s and 1980s hundreds of GS analogs have been produced, either by modification of GS itself, or by chemical synthesis of novel analogs [see reviews by Izumiya *et al.* (2) and Ovchinnikov and Ivanov (6)]. The majority of these studies were aimed at producing a more potent antibiotic by substituting different residues into the backbone of gramicidin S, or creating smaller or larger cyclic structures. Most changes to GS structure, however, have resulted in decreased antibacterial activity. To date, nearly every published result indicates that gramicidin S and its analogs are specific for gram-positive and not gram-negative bacteria, and attribute this to differences in the membrane composition of these microorganisms (2, 7, 8). The mode of action of GS and its analogs is not known, although it is generally accepted that these compounds act on the membrane of sensitive microorganisms, resulting in changes in membrane permeability (9–12). Many biologically active peptides which act on membranes are basic in nature and also have a well defined amphipathic structure. These include both α-helices such as melittin (13) and

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; GS, gramicidin S; HPLC, high-performance liquid chromatography; HEPES, hydroxyethyl piperazine ethanesulfonate; MIC, minimum inhibitory concentration; NMR, nuclear magnetic resonance; NPN, 1-*N*-phenylnaphthylamine; PAM, phenylacetamidomethyl; TFA, trifluoroacetic acid; wt, wild type.

cecropin (14) and cyclic β-sheet structures like defensin and tachyplesin (10, 15, 16).

Owing to the emergence of many medically relevant resistant strains of bacteria, it is clear that new antibiotics are now required to combat these microorganisms. To this end we have synthesized eight GS analogs and have evaluated these peptides as potential antibiotics. One of the central dogmas of GS research has been the long-held belief that GS is active against gram-positive bacteria only. In this paper we present evidence that GS as well as a number of related cyclic analogs are active against both gram-positive and gram-negative bacteria. The fact that this observation has not been made in the past is likely due to the finding that these results are highly dependent on the type of assay used to measure activity.

RESULTS

Antibacterial activity

We have synthesized four linear and four cyclic analogs of GS by replacement of D-Phe in the β-turn portion of the molecule in order better to define the sequence and structure requirements important for antibiotic and hemolytic activity (Table 1). D-Phe was replaced with D-Ser, D-Asn, D-His and D-Tyr and characterized by CD and NMR spectroscopy as reported previously (17, 18). We have also substituted Lys for Orn, as this change has been shown not to alter substantially the antibacterial properties of GS (2). As shown in Table 2, the activity of GS was measured using both an agar assay and a liquid broth assay. For all gram-positive microorganisms tested,

TABLE 1

Sequences, retention time and antibiotic activity of GS β-turn analogs

	Sequence	Retention time (min)[a]	Antibiotic activity (μg/mL)[b]	
			Gram-negative	Gram-positive
Gramicidin S[c]	LFPVOLFPVO	33.5	8	3
Peptide **1**	LYPVKLYPVK	28.7	23	7
Peptide **2**	LSPVKLSPVK	24.3	56	108
Peptide **3**	LNPVKLNPVK	23.7	56	117
Peptide **4**	LHPVKLHPVK	20.2	76	150

[a] Retention time on reversed-phase HPLC.
[b] Average values calculated from microorganisms tested in Table 2 using liquid-broth assays.
[c] O is ornithine.

TABLE 2

Antibacterial and antifungal activity of GS and cyclic GS β-turn analogs

Peptide or antibiotic	Antibiotic activity[a]								
	Gram-negative					Gram-positive			*C. Albicans* CAND105
	E. coli UB1005	*E. coli* DC2	*E. coli* SC9251	*E. coli* SC9252	*P. aeruginosa* H187	*S. aureus* SAP0017	*S. aureus* K147	*B. subtilis* Vernon	
Gramicidin S Agar	>200	200	200	200	>200	3.1	3.1	6.2	200
Gramicidin S Liquid	12.5	3.1	6.2	3.1	12.5	3.1	3.1	3.1	6.2
1 cyclic	25	3.1	25	12.5	50	6.3	12.5	3.1	12.5
2 cyclic	50	6.3	25	100	100	100	200	25	25
3 cyclic	50	6.3	25	100	100	100	>200	50	50
4 cyclic	50	6.3	25	100	200	>200	>200	50	50
Polymyxin B	0.1	0.1	0.1	64	0.3	>64	>64	>64	>64
Gentamicin	0.3	0.3	1	4	0.3	64	2	1	>64
Ceftazidime	0.3	0.03	0.1	4	0.5	2	2	32	>64
Methicillin	>512	0.5	256	128	256	16	0.5	32	>512
Fungizone	>5	>5	>5	>5	>5	>5	>5	>5	1.3

[a] Minimum inhibitory concentration (μg/mL).

antibacterial activity was similar using either assay. For gram-negative bacteria, however, activity was strongly dependent on whether the assay was carried out in solution or in agar. It was found that GS was active against gram-negative microorganisms in the solution-based assay, but inactive against these using an agar-based assay. GS was also found to have antifungal activity when measured using a liquid-broth assay (Table 2).

Using the liquid-broth assay method, the activity of cyclic and linear versions of peptides **1–4** was measured (Table 2). Of the cyclic peptides, peptide **1**, containing the substitution of D-Tyr for D-Phe, showed the greatest antibacterial and antifungal activity, although not as high as GS itself. Replacement of D-Phe with either D-His, D-Ser or D-Asn (peptides **2–4**) resulted in decreased activity. The retention times on reversed-phase HPLC as shown in Table 1 showed a direct correlation with both gram-positive and gram-negative activity, indicating that the most active analogs were those which were most hydrophobic. Gram-positive activity, however, was more sensitive to hydrophobicity, showing a 50-fold difference between the least and most active cyclic peptide, compared to a 10-fold difference for gram-negative microorganisms. All linear peptides were inactive against the strains tested (data not shown).

The antibiotic properties of a number of antibiotics was compared to those of GS as shown in Table 2. Gentamicin, ceftazidime, methicillin and polymyxin B all showed activity against some of the microorganisms tested; however, unlike GS, none was active against all the microorganisms tested and none showed antifungal properties.

Hemolytic activity

We used a liquid-based assay to measure hemolytic activty of the GS β-turn analogs reported in this study (Table 3). The extent of hemolysis by GS was somewhat dependent on the source of red blood cells, with human cells being more susceptible to hemolysis compared to sheep red cells. Of the synthetic analogs, peptide **1** showed the greatest ability to effect lysis of red blood cells. It would appear from the results of peptide **1** that the characteristics which give an analog antibacterial activity also result in hemolytic activity. In other words, hemolytic activity parallels antibacterial activity. It is interesting to note that peptide **4** does not follow this pattern and shows hemolytic activity against human red blood cells, while possessing only weak antibacterial activity. Conversely, peptide **3** shows low hemolytic activity while retaining marginal antibacterial activity. These results indicate that it should be possible to achieve differential activities, i.e. specificity between microorganisms and normal tissues. All linear analogs were found to be non-hemolytic (data not shown).

TABLE 3

Hemolytic activity of GS and GS β-turn analogs

	Hemolytic activity[a]	
	Human red cells	Sheep red cells
Gramicidin S	25	45
Peptide **1**	50	90
Peptide **2**	267	267
Peptide **3**	>267	>267
Peptide **4**	100	200

[a] Concentration required for complete lysis (μg/mL).

Permeabilization of outer membranes to NPN

The hydrophobic fluorescent probe *N*-phenyl-1-naphthylamine (NPN) was used to monitor membrane permeabilization caused by GS and the GS analogs. NPN fluoresces weakly in the presence of bacterial cells under normal conditions. Upon membrane destabilization, however, it can become incorporated into the membrane where it fluoresces strongly in the hydrophobic membrane interior (19). As shown in Fig. 1, all four cyclic analogs as well as GS are able to permeablize the membrane of *E. coli* SC9251 to NPN. The linear analogs were found to have little or no effect on NPN uptake. All analogs were less potent than polymyxin B at destabilizing the membrane.

DISCUSSION

Over the past 30 years, agar-based assays have been used almost exclusively to measure antibacterial activ-

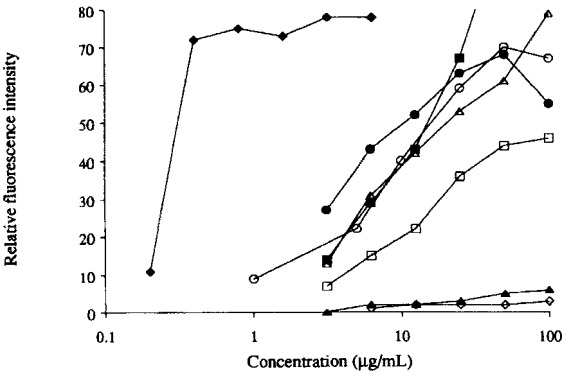

FIGURE 1

Peptide-mediated NPN uptake in *E. coli* SC9251. *E. coli* SC9251 cells were incubated with NPN in the presence of various concentrations of polymyxin B (filled diamonds), GS (open circles), cyclic peptide **1** (filled circles), linear peptide **1** (filled triangles), cyclic peptide **2** (open squares), linear peptide **2** (open diamonds), cyclic peptide **3** (filled squares), or cyclic peptide **4** (open triangles). Linear versions of peptides **3** and **4** are omitted for clarity, and behave as linear peptides **1** and **2** with no effect on NPN uptake. Enhanced uptake of NPN was measured by an increase in fluorescence due to NPN partitioning into the hydrophobic membrane.

ity of GS analogs. It is perhaps a consequence of the exclusive use of agar-based assays that the standard paradigm for GS is that it is a gram-positive specific antibiotic. In the present report we have shown that GS is also active against gram-negative microorganisms when tested using a liquid-based assay. This finding has also been confirmed using several GS-related analogs. A possible explanation for the differences between the two assays may be a reflection of the solubility and diffusion of the analogs in the different media. Alternatively, it may be a function of the water activity in each of the media. GS may permeabilize the membrane in the agar assay, but owing to the low concentration of water, cell lysis is not seen.

It has recently been reported that GS can act on gram-negative bacteria that have been treated with EDTA to permeabilize the outer membrane (20). Following EDTA treatment, *E. coli* cells showed a similar susceptibility to GS as seen with gram-positive cells. Tamaki *et al.* (21) have further shown that GS was in fact adsorbed to *E. coli* cells at levels similar to that of *S. aureus* in a liquid medium, although GS was apparently inactive on *E. coli* cells in an agar assay. In liquid-based assays, Katsu *et al.* (20) found that both melittin and GS were able to cause permeability changes (K^+ efflux) as well as release of phospholipid and lipopolysaccharide in *E. coli* cells, albeit at concentrations slightly higher than those required for similar changes in erythrocytes and *S. aureus*. Hancock and Wong (22) showed that GS was able to permeabilize *P. aeruginosa* outer membranes to nitrocefin, lysozyme and NPN, the latter of which has been confirmed in the present study. The permeabilization was readily reversible by the addition of Mg^{2+} to the medium. These results support an uptake mechanism by the self-promoted uptake pathway (23, 24) in which lipopolysaccharide-bound Mg^{2+} is displaced by polycationic compounds, resulting in outer membrane destabilization. Taken together, membrane interactions of GS with gram-negative cells similar to those with gram-positive cells do occur, and as shown in the present study, either the interactions themselves, or manifestation of membrane damage appear to be dependent on the medium used in the assay.

Consistent with a previous report (25), we found that linear versions of peptides **1–4** showed no antibacterial, antifungal or hemolytic activity. This probably reflects the structural requirements which have been determined empirically, i.e. the requirement for a rigid, amphipathic structure containing basic residues. CD and NMR spectroscopies indicate that these linear analogs contain little structure in solution (unpublished results). The linear analogs were also found to be unable to effect NPN uptake by *E. coli* cells, again reflecting their inability to destabilize membranes.

Of the cyclic analogs, peptide **1** containing D-Tyr in place of D-Phe showed antibiotic activity closest to GS, although this activity was somewhat lower than that of GS. Analogs containing either D-His, D-Ser or D-Asn substitutions were considerably less active than either GS or peptide **1**. We have previously shown that these substitutions (except D-His) do not significantly alter the β-sheet structure of the cyclized decapeptides (17). This result may indicate the need for a large bulky group at this position for activity. Izumiya *et al.* (2) speculated that large bulky D-residues in this position may help stabilize the β-turn to a greater extent. More recently it has been reported that overall hydrophobicity of a series of GS analogs as measured either by retention time on reversed-phase HPLC (21) or by octanol/water partition coefficients (26), correlated well with antibiotic activity. A similar correlation was seen with the cyclic 10-residue analogs reported in this study, with both gram-positive and gram-negative antibacterial activity being correlated with hydrophobicity. Of the two types of bacteria however, gram-positive activity showed a greater dependence on hydrophobicity.

Unlike GS, small-molecule antibiotics such as polymyxin B, gentamicin, ceftazidime and methicillin were found to show specificity in their antibacterial activity in that they were active against some microorganisms and not others. These small-molecule antibiotics also failed to show appreciable antifungal activity. In contrast, GS showed activity against all microorganisms tested as well as antifungal activity, indicating that GS analogs may be good candidates for broad-spectrum antibiotics.

To be useful as an antibiotic, it would be desirable for the analog to possess antibacterial activity with low hemolytic activity. The fact that GS has hemolytic activity and toxicity to liver and kidney has precluded its use as a general antibiotic and has limited its use to a topical antibiotic (27). Unfortunately, of the hundreds of analogs reported in the literature, few have been assayed for hemolytic activity, and no systematic study to remove hemolytic activity while retaining antibacterial activity has been undertaken. However, studies with other cationic peptides have suggested it is possible to dissociate antibacterial activity from anti-eukaryotic cell activity (i.e. toxicity) (28).

We found that hemolytic activity of GS and the cyclic analogs on human red blood cells closely parallelled antibacterial activity. This may be an indication that the mode of interaction of GS is similar between bacterial and erythrocyte membranes. Interestingly, sheep erythrocytes were somewhat less susceptible to hemolysis compared to human red blood cells. This may be explained in part by the increased fragility in our hands of these human red blood cells compared to the sheep red blood cells, possibly due to the somewhat different ages and handling of the two cell types. As demonstrated by

Katsu *et al.* (29), GS lowered the phase transition temperature of acidic membranes more than neutral membranes, indicating a greater destabilization of the former. Furthermore, a number of GS analogs were found to exibit a higher content of β-sheet in the presence of acidic liposomes than in the presence of neutral liposomes, again indicating a greater interaction with negatively charged membranes (9). Our findings may reflect differences in the overall lipid composition of the two red blood cell membranes, and hence the interactions between GS and the membranes may be different.

As has been shown by Kawano *et al.* (15), tachyplesin I exists in an amphipathic structure analogous to that of GS. A recent report (29) has compared antibacterial and hemolytic activity of GS with that of tachyplesin I. The authors found that both antibiotic peptides showed similar antibacterial activity; however, tachyplesin I showed hemolytic activity against human erythrocytes approximately one order of magnitude less than that of GS. These findings would suggest that it is possible to synthesize GS analogs which retain antibacterial activity and have reduced hemolytic activity against human red blood cells. Indeed, Ando *et al.* (9) have reported that GS analogs containing 12, 14 and 18 residues exhibited decreased hemolytic activity against human red blood cells while retaining some antimicrobial activity. In the present study, peptide **4** containing the D-His substitution showed hemolytic activity but low antibacterial activity, and did not follow the general trend of the other analogs in which antibiotic activity parallels hemolytic activity. Apparently, the presence of ionizable groups in the β-turn portion of the molecule results in increased hemolytic activity compared to the aliphatic polar substitutions (Asn and Ser). Peptide **3**, on the other hand, showed a very low level of hemolytic activity while retaining marginal antibacterial activity.

Our findings indicate that GS analogs may be useful as broad-spectrum antibiotics, having activity against both gram-positive and gram-negative bacteria, as well as antifungal activity. GS analogs may therefore have applications as human and veterinary antibiotics. Further development towards a more clinically useful antibiotic will require a systematic study in order to reduce hemolytic activity while retaining the desired antibiotic properties. Our results and those of others indicate that achieving such specificity is possible. We are currently synthesizing a variety of other analogs in order better to define these requirements.

EXPERIMENTAL PROCEDURES

Gramicidin S and NPN were obtained from Sigma (St. Louis, MO). All other chemicals were reagent grade.

Bacterial strains. The following bacterial strains were utilized: *Eschericia coli* UB1005 (wt) and its antibiotic supersusceptible derivative DC2 (30); *Escherichia coli* SC9251 and its polymyxin B resistant mutant pmrA SC9252 (31); *Pseudomonas aeruginosa* H187 (wt) (32); methicillin-sensitive *Staphylococcus aureus* K147 (33); *Staphylococcus aureus* SAP0017, a methicillin-resistant clinical isolate from Dr. A. Chow (University of British Columbia); *Bacillus subtilis*, an environmental wt lab strain. Antifungal activity was tested using a clinical lab isolate of *Candida albicans*.

Peptide synthesis and purification. All peptides were synthesized by solid-phase peptide synthesis using precoupled Boc-Lys(2-ClZ)-PAM resin (Novabiochem, San Diego, CA) on an Applied Biosystems model 430A peptide synthesizer (Foster City, CA) using standard *tert*-butyloxycarbonyl chemistry (34) as reported previously (35). Side-chain protecting groups were 2-ClZ for lysine, 2-BrZ for tyrosine and Bzl for serine. Peptides were cleaved from the resin using anhydrous hydrogen fluoride (20 mL/g resin) in the presence of 10% anisole and 2% 1,2-ethanedithiol for 1 h at -5 °C. Peptides were extracted from the resin with glacial acetic acid and lyophilized. Crude linear peptides were purified by reversed-phase HPLC on a Synchropak RP-4 preparative C8 column (250 × 21.2 mm i.d., 6.5 μm particle size, 300 Å pore size) (Synchrom, Lafayette, IN) using a Beckman System Gold HPLC system (San Ramon, CA). The flow rate was 5 mL/min with a linear AB gradient of 0.25% B/min where solvent A was 0.05% TFA/H_2O and solvent B was 0.05% TFA/acetonitrile. Purity of peptides was verified by analytical reversed-phase HPLC on a Zorbax SB-C8 column (250 × 4.6 mm i.d., 5 μm particle size, 300 Å pore size) (Rockland Technologies, Wilmington, DE) using a Hewlett Packard 1090 chromatograph with a linear AB gradient of 2% B/min and a 1 mL/min flow rate. Identity of peptides was confirmed by time-of-flight mass spectrometry (BIOION-20 Nordic, Uppsala, Sweden).

Cyclization of peptides. Pure linear unprotected peptides were cyclized at a concentration of 2 mg/mL in dichloromethane in the presence of dicyclohexylcarbodiimide, 1-hydroxybenzotriazole and diisopropylethylamine (1.2 equiv. of each) as described elsewhere (17, 18). The progress of cyclization was monitored by both analytical reversed-phase HPLC and mass spectrometry. GS and peptides **1–3** cyclized within 6 h, and peptide **4** did not cyclize completely even after 24 h. Cyclic peptides were purified by reversed-phase HPLC and characterized by NMR spectrscopy to confirm head-to-tail cyclization and to ensure that no racemization had occurred as described (18). Yields of cyclic GS and peptides **1–3** were generally 80% and that of peptide **4** was 15%. The correct

primary ion molecular weights were confirmed by mass spectrometry: GS, calc. 1142.5, obs. 1142.3; peptide 1, calc. 1202.5, obs. 1201.5; peptide 2, calc. 1050.3, obs. 1049.5; peptide 3, calc. 1104.4, obs. 1104.7; peptide 4, calc. 1150.5, obs. 1149.8.

Minimum inhibitory concentration. (a) Liquid-broth method: MICs were determined using a standard microtitre dilution method in LB no salt medium (10 g tryptone and 5 g of yeast extract per L). Briefly, cells were grown overnight at 37 °C in LB and diluted in the same medium. Serial dilutions of antibiotics were added to the microtitre plates in a volume of 100 μL followed by 10 μL of bacteria to give a final inoculum of 5×10^5 CFU/mL. Plates were incubated at 37 °C overnight and MICs determined as the lowest antibiotic concentration that inhibited growth.

(b) Agar method: Agar plates were prepared using LB no-salt medium, 2% agar and 12-fold serial dilutions of antibiotics a maximum of 24 h before assay. The same cell inoculum for the liquid-broth method was used, spotting 10 μL (5×10^4 cells) per plate. Plates were incubated and read as for the liquid-broth method.

Measurement of hemolytic activity. Freshly collected human and sheep blood with heparin was centrifuged to remove the buffy coat, and the erythrocytes obtained were washed three times in 0.85% saline and stored at 4 °C. Serial dilutions of the peptides in saline were prepared in round-bottomed microtitre plates using 100 μL volumes. Red blood cells were diluted with saline to 1/25 packed volume of cells and 50 μL added to each well. Plates were incubated with rocking at 37 °C, and the concentration required for complete lysis determined after 24 h.

Permeablization of outer membranes to NPN. Escherichia coli SC9251 cells were grown to an optical density of 0.5 at 600 nm, centrifuged at 3000*g* for 10 min at 23 °C. Cells were then washed and resuspended to the same optical density in 5 mM HEPES containing 5 mM glucose and 5 mM CCCP. Fluorescence measurements were made using a Perkin-Elmer 650 10S spectrofluorometer, with an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Slit width for both wavelengths was 5 nm. NPN from a 500 mM stock solution in acetone was added to 1 mL of cells in a quartz cuvette to give a final concentration of 10 mM. After the background fluorescence was recorded, 10 μL of peptide was added to the cuvette and fluorescence recorded as a function of time until there was no further increase. A fresh cuvette of cells with NPN was used for each concentration for each peptide. Control experiments were performed to demonstrate that enhanced fluorescence was due to uptake of NPN into cells, as described previously (19, 22, 23).

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