Structure-Activity Relationship Study of Antimicrobial Dermaseptin S4 Showing the Consequences of Peptide Oligomerization on Selective Cytotoxicity*

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To understand how peptide organization in aqueous solution might affect the activity of antimicrobial peptides, the potency of various dermaseptin S4 analogs was assessed against human red blood cells (RBC), protozoa, and several Gram-negative bacteria. Dermaseptin S4 had weak antibacterial activity but potent hemolytic or antiprotozoan effects. K,K20-S4 was 2-3-fold more potent against protozoa and RBC, yet K,K20-S4 was more potent by 2 orders of magnitude against bacteria. K2-S4 had similar behavior as K,K20-S4, but K30-S4 and analogous negative charge substitutions were as active as dermaseptin S4 or had reduced activity. Binding experiments suggested that potency enhancement was not the result of increased affinity to target cells. In contrast, potency correlated well with aggregation properties. Fluorescence studies indicated that K20-S4 and all negative charge substitutions were as aggregated as dermaseptin S4, whereas K-S4 and K,K20-S4 were clearly less aggregated. Overall, the data indicated that N-terminal domain interaction between dermaseptin S4 monomers is responsible for the peptide’s oligomerization in solution and, hence, for its limited spectrum of action. Moreover, bell-shaped dose-response profiles obtained with bacteria but not with protozoa or RBC implied that aggregation can have dramatic consequences on antibacterial activity. Based on these results, we tested the feasibility of selectivity reversal in the activity of dermaseptin S4. Tampering with the composition of the hydrophobic domains by reducing hydrophobicity or by increasing the net positive charge affected dramatically the peptide’s activity and resulted in various analogs that displayed potent antibacterial activity but reduced hemolytic activity. Among these, maximal antibacterial activity was displayed by a 15-mer version that was more potent by 2 orders of magnitude compared with native dermaseptin S4. These results emphasize the notion that peptide-based antibiotics represent a highly modular synthetic antimicrobial system and provide indications of how the peptide’s physico-chemical properties affect potency and selectivity.

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essential defense component of invertebrates and vertebrates, destined to control cell proliferation and invading pathogens (1–3). Antimicrobial peptides display a large heterogeneity in primary and secondary structures but share common features such as amphipathy and net positive charge. These features seem to form the basis for their cytotoxic function. Although their precise mechanism of action is not fully understood, antimicrobial peptides are believed to kill the target cell by destabilizing the ordered structure of the cell membrane via either a “barrel stave” mechanism or a non pore carpet-like mechanism (4–5). Antimicrobial peptides are potentially active against a large spectrum of microorganisms, yet they are generally nontoxic for normal mammalian cells. The molecular basis for this selectivity is also ill defined but believed to result from differences inherent to the lipid composition of target versus nontarget cells, such as membrane fluidity and negative charge density. Indeed, a large body of experimental data demonstrates that factors affecting membrane lytic properties include peptide amphipathy and positive charge distribution (6–12). Moreover, isomers composed of all D-amino acids display identical potency as the all L-amino acid counterparts. This finding represents one of the more fascinating aspects that evolved from the investigation of their mechanism of action, since it implies that if their antimicrobial activity is not mediated by interaction with specific receptors, peptide-based antibiotics could escape some of the mechanisms involved in multidrug resistance.

Dermaseptins are a large family of antimicrobial peptides (28–34 amino acids) expressed in the skin of tree frogs belonging to the phyllomedusinae genus (13–15). They are linear polycationic peptides, structured in amphipathic α-helix in apolar solvents (16) with cytolytic activity in vitro against a broad spectrum of pathogenic microorganisms (bacteria, protozoa, yeast, and filamentous fungi). Using phospholipid liposomes or live cells, the selective antimicrobial action of these peptides was shown to be mediated by selective interaction of the amphipathic α-helix moiety with the plasma membrane phospholipids (7, 10, 12, 17, 18, 21), leading to cell permeabilization. Dermaseptins were shown also to be potent killers of nongrowing and slowly growing bacteria (19), suggesting a potential interest in the eradication of bacteria placed in a dormant state and/or subject to low oxygen tension, which are major factors affecting susceptibility of bacteria to antimicrobial agents, thus leading to drug resistance. More recently, the potential interest of dermaseptins, alone or in combination with mild heating, in killing spoilage yeast was demonstrated (20).

Among natural dermaseptins, dermaseptin S4 is highly toxic to erythrocytes, in contrast to other dermaseptin members, such as dermaseptin S3 (12). Moreover, in Plasmodium
falciparum-infected red blood cells (RBC),
1 dermaseptin S3 selectively killed the intraerythrocytic parasite unlike dermaseptin S4 that was toxic to both the parasite and its host cell. Investigation of the molecular basis for this selective cytotoxicity showed that both dermaseptin S3 and S4 were highly lipophilic as they bound with similar affinity to phosphatidylserine/phosphatidyl choline vesicles and were equally potent in permeabilizing them. However, both NMR and fluorescence methods indicated that dermaseptin S4 was in a higher aggregation state in aqueous solutions, compared with dermaseptin S3. This led to the proposal that the peptide’s aggregation state in solution might be an important factor affecting selective cytotoxicity (12). In the present study, we performed experiments designed to assess the consequences of dermaseptin S4 aggregation in solution. We prepared one set of analogs where Asp replaced Met in position 4, Asn in position 20 or both positions. In another set of analogs, the same positions were substituted with Lys. These analogs were investigated in terms of organization in solution and in terms of cytolytic potency. The results reported, support the notion that peptide aggregation in solution affects considerably cytotoxic properties.

MATERIALS AND METHODS

Peptides—The peptides were synthesized by the solid phase method, applying the Fmoc active ester chemistry as described (14). After removal of the Fmoc from the N-terminal amino acid, the peptide was cleaved from the resin with a mixture of 85:5:5:5 trifluoroacetic acid/para-cresol/H2O/thioanisole (10 mg of resin-bound peptide in a 1-ml mixture). The trifluoroacetic acid was then evaporated, and the peptide was precipitated with ether followed by washing with ether (six times). The crude peptides were extracted from the resin with 30% acetonitrile in water and purified to chromatographic homogeneity in the range of 10% to 95% acetonitrile containing 0.1% trifluoroacetic acid in water. The purified peptides were subjected to amino acid analysis and electrospray mass spectrometry in order to confirm their composition. Peptides were docked as stock solutions in Me2SO and were subjected to amino acid analysis and electrospray mass spectrometry. All other reagents for peptide synthesis and cell cultures were analytical grade. Buffers were prepared using Milli-Q double-distilled water (Millipore Corp.).

Peptide Labeling—Peptide labeling at the N-terminal amino acid with a fluorescent probe was performed by treating 10 μg of resin-bound peptide with 0.8 ml of dimethylformamide containing 20% piperidine in an Eppendorf test tube, in order to remove the Fmoc protecting group of the N-terminal amino acid of the linked peptide. The mixture was agitated for 10 min and then centrifuged, and the supernatant was discarded. The resin-bound peptide was rinsed three times in dimethylformamide before adding a 0.3-ml solution of Lissamine rhodamine B isothiocyanate (1:1000 by weight) to a 0.1-ml volume of the resin-bound peptide in the appropriate medium. The mixture was agitated for 10 min and then centrifuged, and the supernatant was discarded. The resin-bound peptide was rinsed three times in dimethylformamide before adding a 0.3-ml solution of Lissamine rhodamine chloride (10 mg/ml) in dimethylformamide containing 7% (v/v) diisopropylamine. After a 24-h incubation (stirred in the dark at room temperature), the resin-bound peptide was washed thoroughly with dimethylformamide (three times) and diethyl ether/dichloromethane (1:1) and dried at 40 °C (4 h), and then the peptide was cleaved from the resin, precipitated with ether, extracted, and purified as described above.

Erythrocytes—Human blood was rinsed three times in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.3) by centrifugation for 1 min at 2700 × g, and then 2.5 × 108 RBC were suspended in 50 μl of PBS and were added to Eppendorf test tubes containing 200 μl of peptide solutions (serial 2-fold dilutions in PBS), PBS alone (for baseline values), or distilled water (for 100% hemolysis). After incubation (3 h under agitation at 37 °C), samples were centrifuged, and the hemolytic activity was assessed as a function of hemoglobin leakage by measuring the absorbance of 200 μl of supernatant (405 nm). Statistical data were obtained from at least three independent experiments performed in duplicate.

Antimicrobial Assays—Antibacterial activity was assessed against Escherichia coli (TG1), Yersinia kristensenii (ATCC 33639), and Pseudomonas aeruginosa (ATCC 27853) cultured in 2xty medium (16 g/liter trypton, 10 g/liter yeast extract, 5 g/liter NaCl, pH 7.4). Inocula of 106 bacteria/ml were used. The cell populations were estimated by optical density measurements at 620 nm referred to a calibration curve. 100 μl of bacterial suspension were added to 100 μl of culture medium containing no peptide or various peptide concentrations (serial 2-fold dilutions in 96-well plates (Nunk)). Inhibition of proliferation was determined by optical density measurements (620 nm) after the incubation period (3, 16, and 16 h, respectively, for E. coli, Y. kristensenii, and P. aeruginosa) at 37 °C. Antiprotozoan activity was assessed against the promastigote form of a Leishmania major clinical isolate (provided by the parasitology laboratory of Hadassa-Hebrew University, Jerusalem). Inhibition of the proliferation assay was performed by adding 2 × 104 L. major promastigotes (100 μl) to 100 μl of culture medium (RPMI 1640 complemented with 20% fetal calf serum, 1% penicillin, and 1% streptomycin in 96 well plates (Nunk)) containing zero or various peptide concentrations (serial 2-fold dilutions). After the incubation period (3 h, 27 °C), the number of cells was determined by counting an aliquot from each culture on a Neubauer cell counter under a microscope (Olympus IX70).

Reversibility of inhibition was determined by washing the samples (three centrifugation cycles, 15 min each, at 10,000 × g for bacteria and 5,000 × g for Leishmania), resuspending the pellets, and de novo incubation overnight in peptide-free fresh medium.

Binding Experiments—In a separate binding, the supernatants of preparations used for the bioassays were analyzed in HPLC basically as described (12) with the following variation. Cells were added to Eppendorf tubes containing various peptide concentrations in the appropriate culture medium (total volume of 250 μl). After the incubation period, samples were centrifuged, and 100 μl of the supernatant was analyzed by HPLC as described above. Peptide identification was based on retention time and spectral analysis. The amount of peptide present in the supernatant (free peptide) was calculated using standard curves of known concentrations for each peptide. Alternatively, binding of the rhodaminated peptides was assessed by measuring the fluorescence intensity of the supernatant (excitation, 535 nm; emission, 590 nm) compared with standard calibration curves using a Spectrafluor Plus microplate reader (TECAN, Austria). The data were processed with the DeltaSoft 3 analysis program (Princeton University).

Titrations and Analysis of the Rhodaminated Peptides—The aggregative properties of dermaseptins s4 and analogs in aqueous solution were determined using the rhodaminated peptides as described (12). Briefly, 1 μl of free rhodamine or rhodaminated peptide stock solutions (1 mg/ml in Me2SO) were successively added to 1 ml of PBS, and the fluorescence was recorded (excitation, 559 nm; emission, 583 nm) on a Perkin-Elmer fluorimeter. Disruption of the self-quenched fluorescence of the polymers was obtained by measuring the fluorescence 120 min after the addition of 1 unit of proteinase K (Sigma) to a 0.35 μl concentration of each peptide. Alternatively, the rhodaminated peptide was titrated in PBS (1.5 × 105 cells/ml) starting from stock solutions in Me2SO. Fluorescence (excitation, 535 nm; emission, 590 nm) was measured using a Spectrafluor Plus apparatus (TECAN).

Confocal Microscopy—Confocal microscope imaging of samples treated with rhodaminated dermaseptins was performed on nonfixed cells, using an MRC 1024 confocal imaging system (Bio-Rad). The microscope (Axiovert 135M; Zeiss, Germany) is equipped with a × 63 objective (Apodap NA 1.4). For rhodamine excitation, an argon ion laser adjusted at 514 nm (emission, 580 ± 32 nm) was used. 2 series were carried out using 0.18-μm steps between each focal plane.

Hydropathic Index Calculation—The peptide hydropathic index was calculated according to Kyte-Doolittle scale (23), making use of a hydrophobic scale containing hydrophobicity parameters for all 20 amino acids. The scale represents the estimated free energy of transfer of amino acids from an aqueous environment to a hydrophobic environment. It was extracted from experimental solubility measurements of amino acids in ethanol.

7 The abbreviations used are: RBC, red blood cells; Fmoc, Nα-fmocfluorenlymethoxy carbonyl; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; HI, hydropathic index.
RESULTS

Toward better understanding the molecular mechanisms involved in the selective cytotoxicity of peptide-based antibiotics, we used a three-step strategy in which three generations of dermaseptin S4 analogs (substituted, deleted, and combined substituted-deleted analogs) were progressively designed and tested (the peptides and their designations are listed in Table 1). The resulting data are reported separately for each generation of analogs.

Characterization of Generation 1 of Dermaseptin S4 Analogs

This step involved investigation of dermaseptin S4 and six substitution analogs designed to assess the effect of charge on cytotoxicity. Cytotoxicity was assessed against 2 groups of cells selected on the basis of accessibility to their plasma membrane, i.e. cells that allow access (RBC and L. major) and Gram-negative bacteria (E. coli, Y. kristensenii and P. aeruginosa) whose plasma membrane is protected by a complex physical barrier of external membranes. Concomitantly, we investigated the peptides organization in solution, using the same peptides labeled selectively at their N-terminal amino acid with rhodamine.

Cytotoxicity Experiments

Erythrocytes—Incubation of erythrocytes for 3 h in the presence of dermaseptin S4 resulted in massive hemoglobin release corresponding to 50% cell lysis at a peptide concentration of approximately 1 µM (Fig. 1) and 100% RBC lysis at 3 µM (Fig. 5, lower panel). Negative or positive charge monosubstitutions did not affect the peptide’s hemolytic potency. Disubstitutions, on the other hand, had a noticeable effect. Thus, negative charges resulted in reduced hemolytic potency (D4D20-S4 was 4–5-fold less hemolytic), and positive charges resulted in increased hemolytic potency (K4K20-S4 was 2-fold more hemolytic).

Leishmania—As shown in Fig. 1, the antiprotozoan activity of dermaseptin S4 and its analogs practically paralleled that of RBC hemolysis. Thus, dermaseptin S4 was potent in inducing lysis of L. major promastigotes (LC50 2 µM), D4D20-S4 was 5-fold less potent, and K4K20-S4 was 2-fold more potent than the native peptide. Monosubstitutions, positive or negative charges, did not dramatically affect the antileishmanial activity.

Bacteria—Contrary to the hemolytic activity, dermaseptin S4 was revealed to be a weak inhibitor of growth of E. coli, barely achieving 50% inhibition at 40 µM. Negative charge mono- or disubstitutions further weakened the peptide’s antibacterial activity. Interestingly, however, some positive charge substituted peptides displayed potent antibacterial activity. Thus, K4-S4 and K4K20-S4 were 40- and 100-fold more potent, respectively, than the native peptide. K4-S4 on the other hand, was active as dermaseptin S4 (Fig. 1). All remaining peptides were not able to produce 100% bacterial killing up to 100 µM (not shown).

Aggregation in Solution

The results of the proteolytic experiments are shown in Fig. 2. The black columns represent the fluorescence at 583 nm (characteristic of rhodamine emission peak) of the rhodaminated peptides (0.35 µM) in solution, before proteolytic cleavage. At equal concentrations, the rhodaminated peptides displayed clear differences in their fluorescence intensity, especially when compared with Rho(K4K20)S4. The addition of proteinase K caused dequenching of fluorescence (white columns), indicating that the peptides were aggregated to various extents. As expected, when the peptides were cleaved by proteinase K, they exhibited very similar levels of fluorescence. In addition, Fig. 3A depicts a typical result of the fluorescence plot versus the concentration of rhodaminated peptides. The linear nature of the Rho(K4K20)S4 and Rho(K4)S4 plots, at least up to low µM concentrations, suggests that these peptides do not change their state in this concentration range.

Combining these results, it can be concluded that the peptides tend to aggregate in aqueous solutions to different extents. The aggregational state of dermaseptin S4 was not reduced by negative charge substitutions. In contrast, positive charge monosubstitution in position 4, but not in position 20, reduced the peptide’s aggregation state. Peptide aggregation was further reduced in disubstituted K4K20-S4. To verify whether rhodamination affected the peptides cytotoxic properties, the rhodaminated versions of S4, K4-S4, and K4K20-S4 were examined in parallel to the nonlabeled versions. No significant difference was observed in their antiprotozoan or he-

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TABLE I

Structural and biological properties of dermaseptin S4 and analogs

| Peptide | Amino acid sequence | Charge | HT | IC50 µM | LC50 µM | RSI |
|---------|---------------------|--------|----|---------|---------|-----|
| S4 | ALWNTLLKVKLAAKALNAVLVGANA | 4 | 28.9 | 40 ± 5 | 1.4 ± 0.2 | 1 |
| D4-S4 | ----D---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- ---- 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Fig. 1. Cytotoxicity of dermaseptin S4 and its substitution analogs against human RBC (white columns) L. major (gray columns), and E. coli (black columns) after a 3-h exposure in culture medium at 37 °C. LC50 is defined as the lowest peptide concentration that induced 50% lysis of erythrocytes or 50% killing of L. major promastigotes compared with untreated control cells. IC50 is the peptide concentration that induced 50% bacterial growth inhibition. The arrow indicates that the IC50 was not reached up to 50 μM. Statistical data was obtained from at least three independent experiments performed in duplicates.

![Peptide](image)

**Fig. 2.** Typical proteolytic outcome of the rhodaminated peptides in PBS. The **black columns** show the initial fluorescence of rhodaminated dermaseptin S4 (Rho-S4) or the substitution analogs at 0.35 μM. The **white columns** show the final fluorescence, 120 min after the addition of proteinase K.

lytic activity of K4K20-S4 were not reduced at high peptide concentrations (Fig. 4, D and E, respectively). A possible explanation of these results is given under “Discussion.”

**Fluorescence Microscopy**

Observation under microscope of RBC and E. coli cultures treated with rhodaminated dermaseptin S4 (3 μM) revealed that both types of cells were labeled at their periphery. Typical fluorescence images of treated RBC (ghosts) and two nascent bacteria (forming the shape of the number 8) are shown in Fig. 7, A and B, respectively. The labeling pattern suggested no intracellular fluorescence but displayed a regular fluorescent contour, punctuated with intensely fluorescent spots, which, in bacteria, generally localized to the polar caps and/or at the junction point. Contrasting with this general pattern of fluorescence, the peptides with the highest inclination for aggregation (especially K20-S4), often displayed larger fluorescent spots that seemed to project out of the cell. Analysis by confocal microscopy supported the possibility of partial extracellular localization of Rho-K20-S4 oligomer as depicted in Fig. 7, C1–C5.

**Binding Experiments**

The relationship between peptide cell affinity and cytotoxicity was investigated by analysis of the culture supernatants after the specified incubation period. Thus, at peptide concentration of 5 μM (1 nmol), no free peptide could be detected in the supernatants of RBC, suggesting that nearly 100% of dermaseptin S4 was cell-bound. Observation of the cells treated with this concentration showed that no characteristic shape of RBC can be found; instead, only flat “ghosts” were present. Yet, incubation of RBC with more than 10-fold higher peptide concentrations still resulted in >85% binding. Fig. 8A depicts the binding of native S4 to RBC, based on HPLC analysis. Binding based on the fluorescent peptides was strictly similar (not shown). The plot revealed that peptide binding to RBC is practically linear over a wide range of concentrations. Fig. 8B shows the dermaseptin S4 binding to E. coli. Surprisingly, S4 bound between 80 and 100% over a wide range of concentrations although the peptide displayed weak antibacterial activity. At a sample concentration of 70 μM, about 90% of dermaseptin S4 (12.6 ± 0.8 nmol) was estimated to be bound to E. coli. All of the analogs, whether they displayed higher or lower activity, were also estimated to bind between 11.9 and 12.6 nmol (not shown).

An isomeric version of K4K20-S4 composed with all L-amino acids yielded the same results as the l-amino acid version with respect to binding, hemolysis, and antibacterial activity, suggesting that the observed properties were not biased by differential susceptibility to proteolytic enzymes (not shown).
Characterization of Generation 2 of Dermaseptin S4 Analogs

To identify the molecular elements in the structure of dermaseptin S4 that are necessary for cytotoxicity, we prepared a set of deletion analogs in which the native sequence was progressively truncated by four residues at a time, either from the N- or the C-terminal end or from both ends. Activity of these analogs was compared by determining cytotoxicity in terms of the minimal inhibitory concentration defined as the lowest peptide concentration that induced 100% irreversible growth inhibition of Gram-negative bacteria *E. coli*. When the minimal inhibitory concentration was not achieved at high concentrations, activity was compared in terms of 50% growth inhibition (IC50). Selectivity was assessed by comparing antibacterial activity to hemolytic activity in terms of 100 and 50% lytic concentration (LC50).

**Cytotoxicity**

**Antibacterial Activity**—Stepwise deletion of the C-terminal 4–12 residues had the effect of enhancing antibacterial activity. For instance, the 16-mer analog S4-(1–16) was twice as active, and the 20-mer analog S4-(1–20) was 4-fold more active than the native peptide (Fig. 5, upper panel). Moreover, unlike the native peptide, these analogs were able to induce 100% growth inhibition (minimal inhibitory concentration of 30 and 12 μM, respectively). Further C-terminal deletions had the effect of reducing activity; the IC50 of S4-(1–12) reached 53 μM, and the minimal inhibitory concentration was not observed up to 70 μM. Surprisingly, deletion of the first four N-terminal residues yielded a peptide S4-(5–28) that was 11-fold more active than dermaseptin S4 (IC50 3.5 μM). Further deletions, however, yielded inactive analogs; IC50 of S4-(9–28) and S4-(13–28) were not reached at 80 μM. Simultaneous deletion of both hydrophobic domains, S4-(5–16), leaving the central sequence bearing all of the positive charges, also resulted in an inactive peptide (IC50 > 80 μM).

**Hemolytic Activity**—As shown in the lower panel of Fig. 5, deletion of up to eight C-terminal residues (yielding S4-(1–20)) had the effect of reducing the peptide's hemolytic potency by 4-fold (LC50 = 5 μM). Further deletions led to gradual loss of hemolytic activity; *i.e.* S4-(1–16) and S4-(1–12) were, respectively, 16- and 71-fold less active than dermaseptin S4. In contrast to antibacterial activity, stepwise N-terminal deletions led to progressively less hemolytic peptides. Thus, S4-(5–28), S4-(9–28), and S4-(13–28) were, respectively, 12-17-, and 29-fold less hemolytic than dermaseptin S4. Finally, S4-(5–16) produced virtually no hemolysis up to 80 μM.

**Binding Experiments**

Fig. 8A depicts the binding profile of S4-(1–12) and S4-(5–16) to RBC. Surprisingly, these short analogs were found to bind...
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Fig. 5. Dose-response profiles for growth inhibition of E. coli (upper panel) and hemolytic activity (lower panel) of dermaseptin S4 and the first generation analogs after a 3-h exposure in culture medium at 37 °C. Stars, S4; filled circles, S4-(1–20); empty circles, S4-(1–16); filled rectangles, S4-(1–12); empty rectangles, S4-(5–28); filled triangles, S4-(9–28); empty triangles, S4-(13–28); empty diamonds, S4-(5–18). Note that 100% hemolysis refers to hemoglobin release caused by water.

Fig. 6. Dose-response profiles for growth inhibition of E. coli (upper panel) and hemolytic activity (lower panel) of dermaseptin S4 and the second generation analogs after a 3-h exposure in culture medium at 37 °C. Stars, S4; filled circles, K4-S4-(1–16); empty circles, K4-S4-(1–16)amide; filled rectangles, K4-S4-(1–15)amide; empty rectangles, K4-S4-(1–15)amide; filled triangles, K4-S4-(1–10)amide.

RBC to a considerable extent despite having virtually no hemolytic activity. Fig. 8B depicts their binding profile to E. coli. As observed with RBC, S4-(1–12) and S4-(5–16) bound between 80 and 100% over a wide range of concentrations although they displayed weak or no antibacterial activity. This indicated that binding does not necessarily lead to cytotoxicity but merely reflects the peptide’s lipophilic properties, as argued under “Discussion.”

Characterization of Generation 3 of Dermaseptin S4 Analogs

The design of the third generation of dermaseptin S4 analogs was inspired by the results obtained with the deleted analogs. Thus, starting with dermaseptin S4-(1–16), we searched for the shortest peptide whose positive charge/peptide length ratio was most favorable for potent yet selective antibacterial activity. Assessment of these peptides proceeded as described above.

Cytotoxicity

Antibacterial Activity—Substitution of the methionine with lysine in position 4 of dermaseptin S4-(1–16) enhanced antibacterial activity, since K4-S4-(1–16) was 8-fold more potent than its counterpart and 16-fold more potent than native dermaseptin S4 (Fig. 6, upper panel). Moreover, neutralization of the negative charge at the C terminus, by conversion of the carboxylate to a carboxamide, yielded a peptide derivative that displayed a further increase in potency; K4-S4-(1–16)amide (where “a” represents amide) was approximately 6 times more potent than K4-S4-(1–16). Stepwise elimination of the C-terminal residues starting from K4-S4-(1–16)amide resulted in the following: a 15-mer analog with similar antimicrobial potency (IC50 = 0.38 μM); a 13-mer analog, K4-S4-(1–13)amide, slightly less potent (IC50 = 1.35 μM); and a 10-mer analog, K4-S4-(1–10)amide, displaying considerably less antibacterial activity (IC50 = 40 μM). Growth inhibition was irreversible for all samples displaying 100% growth inhibition (not shown). This included all peptides but the native peptide and the four analogs S4-(1–12), S4-(5–16), S4-(9–28), and S4-(13–28), indicating that most S4 analogs were not simply involved in a static inhibitory effect, but they were actually able to kill the bacteria. In addition, the isomer versions of K4-S4-(1–15)amide, K4-S4-(1–13)amide, and K4-S4-(1–10)amide composed with all D-amino acids yielded similar results as the all L-amino acid counterparts, indicating that the observed activity of these peptides was not biased by susceptibility to E. coli proteolytic enzymes.

Hemolytic Activity—All of the generation 3 analogs had weaker hemolytic activity compared with dermaseptin S4, although their antibacterial activity was equal or enhanced (Fig. 6, lower panel). It is interesting to note that K4-S4-(1–10)amide, whose antibacterial activity was comparable with that of dermaseptin S4 (IC50 = 40 μM), displayed no hemolytic activity up to 100 μM. Fluorescence confocal microscopy imaging of cell-bound dermaseptins is shown in Fig. 7.

Binding Experiments

Fig. 8A shows the binding profile of selected peptides to RBC, based on HPLC analysis. The weakly hemolytic analog K4-S4-(1–15)amide bound less than S4, and the inactive analog K4-S4-(1–10)amide bound the least. Fig. 8B shows the binding profile of the same peptides to E. coli. Whereas K4-S4-(1–10)amide bound weakly, 100% of K4-S4-(1–15)amide was bound to bacteria at 5 μM (1 nmol). Still higher binding was observed with increasing K4-S4-(1–15)amide concentrations, although all bacteria are killed at lower concentrations. The all D-amino acid versions of K4-S4-(1–15)amide and K4-S4-(1–10)amide yielded similar results as the all L-amino acid counterparts, with respect to their binding to RBC and...
bacteria, suggesting that the observed properties were not biased by differential susceptibility to proteolytic enzymes (not shown).

Finally, the aggregational properties of three short analogs, K4-S4-(1–16)a, K4-S4-(1–15)a, and K4-S4-(1–13)a, were investigated as described above. Fig. 9 depicts the fluorescence plots...
Effect of Aggregation—Dermaseptin S4 and its derivatives aggregate in aqueous solutions to different extents. For example, the aggregational state of dermaseptin S4 was not reduced by negative charge substitutions in peptide D4-S4, D20-S4, or K20-S4, have an increased relative selectivity index compared with S4.

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DISCUSSION

Properties of Dermaseptin S4-derived Peptides—To identify peptides derived from dermaseptin S4 with a reduced hemolytic activity and with an improved antimicrobial activity and to identify the factors, such as charge, hydrophobicity, and aggregation, that influence these characteristics in solution, substitution, deletion, and combined substitution/deletion, peptides derived from dermaseptin S4 were prepared. As summarized in Table I, nearly all of the dermaseptin S4-derived peptides possess some antibacterial activity, and most possess a significantly reduced hemolytic activity. The peptide concentration causing 50% lysis of RBC (LC_{50}) and the peptide concentration causing 50% growth inhibition of *E. coli* (IC_{50}) were measured. The relative selectivity index, *i.e.* the ratio of LC_{50}/IC_{50}, of a given derivative relative to that of dermaseptin S4, was calculated. The relative selectivity index is thus a combined measurement of two preferred characteristics of a peptide: high LC_{50} (reduced hemolysis) and low IC_{50} (increased antimicrobial activity). All of the peptides, apart from three of the four full-length singly substituted peptides, D4-S4, D20-S4, and K20-S4, have an increased relative selectivity index compared with S4.

![Image](93x431 to 253x729)

Fig. 8. Binding of rhodaminated dermaseptin S4 to RBC (A) and *E. coli* (B). Peptide binding was estimated by HPLC analysis of the culture supernatants as described (12). The amount of free peptide was calculated using standard curves of known concentrations for each peptide. Stars, S4; triangles, S4-(1–12); empty circles, S4-(5–16); rectangles, K, S4-(1–15)amide; filled circles, K, S4-(1–10)amide.

versus the concentration of the rhodaminated peptides. The shape of the plots obtained suggested that unlike dermaseptin S4, the short analogs did not change their state as concentration increased, indicating that they did not conserve the aggregational properties of the parent peptide.

FIG. 8. Binding of rhodaminated dermaseptin S4 to RBC (A) and *E. coli* (B). Peptide binding was estimated by HPLC analysis of the culture supernatants as described (12). The amount of free peptide was calculated using standard curves of known concentrations for each peptide. Stars, S4; triangles, S4-(1–12); empty circles, S4-(5–16); rectangles, K, S4-(1–15)amide; filled circles, K, S4-(1–10)amide.

**FIG. 9.** Second order curve fit of rhodaminated peptides in PBS solution showing the dose-response relationships. Stars, S4; rectangles, K, S4-(1–16)-amide; circles, K, S4-(1–15)amide; triangles, K, S4-(1–13)amide.

D4D20-S4. However, positive charge monosubstitution in position 4, K, S4, but not in position 20, reduced the peptide’s aggregation state. Peptide aggregation is further reduced in disubstituted K, K20-S4.

Unlike other natural dermaseptins, where the positive charges are more or less regularly spaced along the peptide sequence, dermaseptin S4 has four positive charges concentrated in the middle, flanked by two hydrophobic stretches. If one (or both) of the extremities is involved in hydrophobic interactions with other peptides, insertion of positive charges might force depolymerization due to charge repulsion.

Titration of the rhodaminated peptides in PBS showed that the doubly substituted peptide Rho(K, K20)S4 was clearly less aggregated than native dermaseptin S4. Moreover, the fact that of the two corresponding singly substituted peptides Rho(K20)S4 is aggregated but Rho(K)S4 is not further indicates that the N-terminal hydrophobic stretch is primarily responsible for aggregation. Deletion experiments performed on the sequence of dermaseptin S4, where the hydrophobic stretches were sequentially truncated, confirmed this view. In accordance with this view, the effect of negative charge insertion within a high positive charge environment is likely to be negligible or even to encourage aggregation.

The factors believed to influence cytotoxicity include peptide size, amphipathy, and positive charge distribution. The peptides’ positive charge is consistent with their affinity for microbial membranes bearing negative charges. The peptides’ amphipathy is consistent with their capacity to intercalate into membranes. These actions are likely to be facilitated by their small size. A comparison of the data of antimicrobial activity, hemolytic activity, and aggregation state of the peptides of the present study indicates that peptide organization in solution represents another factor. The peptides’ aggregation profile correlates very well with their antibacterial potency. Whereas aggregated peptides are weakly active against Gram-negative bacteria, the less aggregated peptides are highly active.

It is believed that peptide aggregation in solution affects the cytotoxicity induced by, and hence the selectivity of, antimicrobial peptides. This may be of utmost importance in the design and assessment of potent and/or selective peptide-based antimicrobials. The view that hydrophobic interactions involving primarily the N-terminal sequence of dermaseptin S4 (and to a lesser extent the C terminus) are responsible for the peptide’s aggregation in solution is confirmed by the results obtained with the deletion derivatives. In this respect, dermaseptin S4 presents a surprisingly and unexpectedly different behavior from the other dermaseptins, since shortening the S4 N-termi-
nal domain by four residues actually enhanced antibacterial activity while simultaneously reducing the hemolytic activity. An explanation for this behavior might reside in a particular sequence motif of the N terminus.

Peptides whose N terminus is moderately hydrophobic had reduced hemolytic activity, such as in S4-(5–28) (whose N terminus is TLL-) or all of the deletion derivatives as well as all natural dermaseptins (whose N terminus is ALW-). In contrast, peptides whose N terminus is highly hydrophobic are hemolytic. Such examples include the native dermaseptin S4 and its derivative S4-(1–20), whose N terminus is ALWMTLL-.

The dermaseptin derivative peptides of the present investigation were unexpectedly found to be particularly susceptible to N-terminal modifications. If the N-terminal sequence is involved in peptide aggregation, its deletion probably reduces aggregation or even produces monomeric peptides. The N-terminal deletion peptide S4-(5–28) and other deletion derivatives displayed potent antibacterial activity and weak hemolytic activity. In addition, the fluorescence plots of rhodaminated peptides indicated that the highly antimicrobial and weakly hemolytic peptides K4-S4-(1–15)a and K4-S4-(1–13)a were much less aggregated than dermaseptin S4. In contrast, S4-(1–20) is probably still aggregated, which may explain why this peptide displayed strong hemolytic activity.

It was previously shown that the length of various synthetic model polycationic peptides influences their hemolytic properties; shorter peptides display reduced hemolytic activity, accompanied by reduced antimicrobial potency, compared with their longer derivatives (8, 9). In contrast, the present shortened dermaseptin S4 derivatives display reduced hemolytic activity combined with enhanced antibacterial activity.

Effect of Charge Versus Hydrophobicity—The relative force of two parameters affecting cytotoxicity, i.e. charge and hydrophobicity, was assessed. With respect to the issue of selective toxicity between bacteria and RBC, our data showed that, surprisingly and unexpectedly, the hydrophobicity, not the charge, is the critical parameter for selectivity. Comparison of the peptides’ hydropathic index (HI) and charge with their hemolytic and antimicrobial activities supports this view. These properties of dermaseptin S4 and derivatives are listed in Table I.

By way of illustration, a comparison can be drawn between dermaseptin S4 and the C-terminally deleted derivatives S4-(1–20) and S4-(1–16), which have an identical net positive charge. Dermaseptin S4 (HI 28.9) has potent hemolytic activity and weak antibacterial activity; S4-(1–20) (HI 15.2) has somewhat reduced hemolytic activity but enhanced antibacterial activity; and S4-(1–16) (HI 11.3) even less hemolytic activity and further enhanced antibacterial activity. The relative selectivity index of the latter peptides rose by a factor of 16 and 33, respectively.

By way of another illustration, a comparison can be drawn between the three derivatives S4-(1–16), K4-S4-(1–16) and K4-S4-(1–16)NH2. These peptides are all the same length of 16 amino acids, but they possess increased charge (4, 5, and 6 respectively) and reduced HI (11.3, 5.5, and 5.5, respectively). S4-(1–16) has weak hemolytic activity and relatively weak antibacterial activity. Increasing its charge from 4 to 5 by substitution with lysine (K) reduces its hydropathic index from 11.3 to 5.5. This results in a weakened hemolytic activity and an enhanced antibacterial activity; the relative selectivity index rose from 33 to 651. Further increasing the charge of K4-S4-(1–16) from 5 to 6 by amidation, which does not modify the hydropathic index, results in enhancing both activities evenly by 6-fold. Based on these observations, we conclude that while hydrophobicity contributes to antimicrobial versus hemolytic specificity, charge contributes to potency.

The complex relationships between hydrophatic index and positive charge contributions to the activity of dermaseptin S4 may be summarized as follows. 1) A combination of low hydrophatic index (about <10) with high positive charge (about >4) is consistent with low hemolytic activity and high antibacterial activity, e.g. K4-S4-(1–16). 2) A combination of high hydrophatic index (about >20) and low positive charge is consistent with high hemolytic activity and low antibacterial activity, e.g. D20-S4. 3) A combination of low hydrophatic index and low positive charge is consistent with general reduced cytotoxicity, e.g. S4-(1–12). 4) A combination of high hydrophatic index and high positive charge is consistent with general enhanced cytotoxicity, e.g. K4K20-S4.

Intermediate properties balance between the extremes mentioned above, taking into consideration that these relationships are highly susceptible to peptide organization in solution. Thus, although K4K20-S4 combines a high hydrophatic index with a high positive charge, its high aggregation state in solution is probably responsible for obstructing its antibacterial activity, whereas the aggregation state of D20-S4 does not hamper its hemolytic activity, and the peptide behaves as predicted.

In conclusion, we have demonstrated selectivity reversal in the activity of dermaseptin S4 by identifying the topological domains that are responsible for hemolytic activity. Modification of these domains in terms of amphipathy and charge led to the design of potent and selective short antibacterial peptides. Thus, the present study showed the dermaseptins to behave as a modular synthetic antimicrobial system and further suggests that the spectrum of action of other peptide-based antibiotics may be tailored specifically to adapt to target cells and avoid undesirable toxicity by rational manipulations of their sequence.

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