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Synthetic histatin analogues with broad-spectrum antimicrobial activity

Eva J. HELMERHORST*[1], Wim VAN 'T HOF*, Enno C. I. VEERMAN*, Ina SIMOONS-SMIT† and Arie V. NIEUW AMERONGEN*

*Vrije Universiteit, ACTA, Department of Oral Biochemistry, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands, and †AZVU, Department of Clinical Microbiology, de Boelelaan, Amsterdam, the Netherlands

Histatins are salivary histidine-rich cationic peptides, ranging from 7 to 38 amino acid residues in length, that exert a potent killing effect *in vitro* on *Candida albicans*. Starting from the C-terminal fungicidal domain of histatin 5 (residues 11–24, called dh-5) a number of substitution analogues were chemically synthesized to study the effect of amphipathicity of the peptide in helix conformation on candidacidal activity. Single substitutions in dh-5 at several positions did not have any effect on fungicidal activity. However, multi-site substituted analogues (dhvar1 and dhvar2) exhibited a 6-fold increased activity over dh-5. In addition, dhvar1 and dhvar2 inhibited the growth of the second most common yeast found in clinical isolates, *Torulopsis glabrata*, of oral- and non-oral pathogens such as *Prevotella intermedia* and *Streptococcus mutans*, and of a methicillin-resistant *Staphylococcus aureus*. In their broad-spectrum activity, dhvar1 and dhvar2 were comparable to magainins (PGLa and magainin 2), antimicrobial peptides of amphibian origin. Both the fungicidal and the haemolytic activities of dhvar1, dhvar2 and magainins increased at decreasing ionic strength.

INTRODUCTION

Candidiasis is an infectious disease caused mainly by *Candida albicans*. The development of oral candidiasis is seldom observed in healthy subjects but seen frequently in people with reduced saliva output (xerostomia), in people wearing dentures and in immunocompromised patients [1]. The underlying pathogenesis of this oral infection is not well understood. The discovery of histatins, salivary proteins exhibiting fungicidal and fungistatic properties [2–4], revealed a mechanism by which the colonization and subsequent oral infection with *Candida* could be controlled. Histatins and other antimicrobial peptides with broad-spectrum activity, including defensins and magainin-like peptides, have been identified in the oral cavity and could possibly contribute to the non-immune host defence system [5–7]. The common features of these host defence peptides are their basic nature, due to the presence of multiple arginine and lysine residues, and the proposed mechanism of pore formation in the membrane of the target organism [8–10]. Histatins are a group of small cationic histidine-rich proteins secreted by the parotid salivary gland and to a smaller extent by the submandibular salivary glands [11]. These peptides possess a number of antifungal and antibacterial properties, such as growth-inhibitory effects on *C. albicans* [4,11,12], inhibition of the conversion of *C. albicans* yeast growth into hyphal growth [13,14], growth inhibition of *Streptococcus mutans* [15] and inhibition of a protease produced by *Porphyromonas gingivalis* [16].

Three major histatins, histatins 1, 3 and 5, have previously been purified and characterized [11,17]. CD, IR and NMR studies on residues 9–24 of histatin 5 indicated that histatins exhibit random coil conformations in aqueous solution, but that α-helix conformations are preferred in trifluoroethanol and DMSO [18,19]. On the basis of these studies a model of pore formation has been deduced for the mechanism of killing. Inducibility of amphipathic helical structures would allow the incorporation of the peptide into the membrane of the target organism and the formation of a water-filled pore, leading to cell death [9]. The loss of viability of *C. albicans* on administration of histatins has been found to be concomitant with loss of K^+ ions from exposed yeast cells, suggesting that these peptides can alter membrane permeability and cause membrane damage [4]. Structure–function studies on other pore-forming antimicrobial peptides revealed that amphipathicity and the α-helical content are important conditions for antimicrobial activity [8,20–22].

In the present study we chemically synthesized substitution analogues of histatins with the intention of increasing the lateral amphipathicity of the helical conformation, and evaluated the effect on antifungal and antibacterial activity. For comparison, two antimicrobial peptides of amphibian origin, PGLa (peptide with N-terminal glycine and C-terminal leucine amide) and magainin 2, were included. Magainins and histatins do not share amino acid sequences, but both are expected to form amphipathic α-helices and to kill their target organism by pore formation [8]. Polylysine of comparable chain length (15-mer) was tested to evaluate the importance of positive charges on fungicidal activity. Unlike histatins and magainins, short polylysine peptides do not adopt helical conformations in liposome vesicles [23] and have no amphipathic character. The lytic activities of these peptides against fungal cells and cells from mammalian origin (erythrocytes) were compared.

MATERIALS AND METHODS

Strains and growth conditions

C. albicans 315 (ATCC 10231) and *Torulopsis glabrata* 359 (ATCC 90030) were grown aerobically at 30 °C on Sabouraud dextrose agar (SDA, Oxoid, Hants., U.K.), *Streptococcus mutans* HG 982, *S. mutans* HG 1003, *S. mutans* Ingbritt, *S. sanguis* (clinical isolate from blood) and *Actinobacillus actinomycetemcomitans* Y4 (ATCC 43718) in air/CO_2 (19:1) at 37 °C, *Fusobacterium nucleatum* (ATCC 10953), *Veillonella parvula* (ATCC 17745) and *Prevotella intermedia* (T588) anaerobically at

Abbreviations used: Fmoc, fluoren-9-ylmethoxycarbonyl; PGLa, antimicrobial peptides of amphibian origin (peptide with N-terminal glycine and C-terminal leucine amide); SDA, Sabouraud dextrose agar; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; PBB, potassium phosphate buffer.

[1] To whom correspondence should be addressed.
[2] † AZVU, Department of Clinical Microbiology, de Boelelaan, Amsterdam, the Netherlands

37 °C, and *Staphylococcus aureus* (methicillin resistant, MRSA; clinical isolate from perineum) on Schaedler F0 agar (Oxoid) containing 1% (v/v) haemin and 7% (v/v) horse blood (Biotrading, Mijdrecht, the Netherlands).

C. albicans and *T. glabrata* were cultured aerobically in tryptic soy broth (Becton Dickinson, Cockeysville, MD, U.S.A.), *F. nucleatum*, *V. parvula* and *P. intermedia* anaerobically in BM1 medium, and *A. actinomycetemcomitans* in tryptic soy broth containing 0.5% yeast extract (Difco, Detroit, MI, U.S.A.) and 0.1% $NaHCO_3$, pH = 7.2. *S. mutans* and *S. sanguis* were cultured in Todd Hewitt medium (Oxoid) in air/CO_2 (19:1), and *Staph. aureus* was cultured aerobically in Brain Heart Infusion (BHI, Difco).

Induction of germ tube formation

C. albicans was grown in Sd medium [0.17% Yeast Nitrogen Base without amino acids and $(NH_4)_2SO_4$ (Difco)/0.5% $(NH_4)_2SO_4$/0.25% succinic acid (pH 4.5)/2% (w/v) glucose] at 28 °C. To induce germ tube formation, yeast cells in the exponential growth phase ($1 < D_{530} < 2$) were collected by centrifugation and washed once in doubly distilled water. The yeast cells were then resuspended in a modified Sd medium containing 0.25% Mes, pH 6.7, and 10 mM *N*-acetylglucosamine instead of succinic acid and glucose respectively. These cell suspensions were shaken for 2 h at 37 °C to develop germ tubes (hyphae).

Peptides

Histatin derivatives were synthesized by the 'T-bag' method, adapted for fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry, as described elsewhere [24,25]. T-bags (3.5 cm × 3.5 cm) were made by sealing 64 μm polypropylene mesh (Chicopee Industries, Gainesville, GA, U.S.A.). *p*-Benzyloxybenzyl alcohol resins, with the first *N*-Fmoc-protected amino acids already attached, were included inside the T-bags, in such quantities that each T-bag contained 50 μmol of the first amino acid.

Coupling reactions were performed for 1 h at ambient temperature in fresh Pro Analyse-grade dimethylformamide, from which the degradation product dimethylamine was removed by sparging with nitrogen. A 10-fold excess of *N*-Fmoc-protected amino acid was used with respect to the loading of the first amino acid on the resin. As coupling reagent a 10-fold excess of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was used; to prevent racemization a 10-fold excess of 1-hydroxybenzotriazole (Fluka, Buchs, Switzerland) was added; and for activation a 13-fold excess of *N*,*N*-di-isopropylethylamine (Aldrich, Bornem, Belgium) was used. After each coupling step the T-bags were extensively washed with dimethylformamide, dichloromethane and propan-2-ol. TBTU, the preloaded resins and the amino acid derivatives were purchased from Novabiochem/Calbiochem (Läufelfingen, Switzerland).

Semi-permanent side chain protection of arginine was achieved with the N^G-2,2,5,7,8-pentamethylchroman-6-sulphonyl group, of histidine and cysteine with the triphenylmethyl group, and of tryptophan with the t-butoxycarbonyl group. Asparagine and glutamine were used without side chain protection. For the other amino acids the standard t-butoxycarbonyl/t-butyl side chain protection strategy was followed.

After completion of the sequence, cleavage from the resin and simultaneous side chain deprotection were achieved with a mixture of 5% (v/v) thioanisole, 5% (w/v) phenol, 5% (v/v) water and 85% (v/v) trifluoroacetic acid (5 ml per T-bag) at ambient temperature within 4 h. The volume was reduced to 1 ml by evaporation of most of the trifluoroacetic acid with a stream of nitrogen. On addition to ice-cold ether (15 ml) the peptides precipitated. The T-bags were extracted twice with acetic acid (3 ml per T-bag) and these extracts were added to the ether phase. After the ether had been decanted, the combined precipitates were triturated with ether and dissolved in water. After extraction with ether to remove the last traces of scavengers the peptides were freeze-dried.

For purity analysis reverse-phase HPLC was performed with an Applied Biosystems C_{18} column (ODS, 220 mm × 4.6 mm) in conjunction with a Spectroflow 480 HPLC system and two Spectroflow 400 pumps (ABI Kratos Analytical Division). Samples were monitored at 214 nm with a 783 programmable absorbance detector (ABI Applied Biosystems). Buffer A consisted of 0.1% (v/v) trifluoroacetic acid in water and buffer B of 0.1% (v/v) trifluoroacetic acid in acetonitrile. The gradient was run from 0 to 100% buffer B at a flow rate of 1 ml/min.

Synthetic PGLa (95% pure) was kindly provided by Professor H. V. Westerhoff (Department of Microbial Physiology, Vrije Universteit, Amsterdam, The Netherlands). Synthetic magainin 2 was a gift from Dr. B. J. Appelmelk (Department of Medical Microbiology, Vrije Universiteit, Amsterdam, The Netherlands). Poly-L-lysine (degree of polymerization, 15 residues; approximate molecular mass based on viscosity determination, 3000 Da) was obtained from Sigma (St. Louis, MO, U.S.A.).

All peptides were water-soluble and dissolved in 10 mM potassium phosphate buffer (PPB), pH 7.0, to a final concentration of 2 mg/ml. The exact concentrations were determined by amino acid analysis. Stock solutions were stored at −80 °C in Eppendorf vials.

Killing assay

The fungicidal assay was performed largely by the method of Raj et al. [18]. In 96-well culture plates (Greiner), peptides were diluted 2-fold serially in 1 mM PPB, pH 7.0, starting from 250 μg/ml. Columns 1 and 2 served as growth controls (no peptide added). Before the experiment, *C. albicans* 315 was cultured from glycerol on SDA and maintained for at most 1 week. Several yeast colonies were picked up from the plate and suspended in 1 mM PPB, pH 7.0, to yield a suspension of 10^7 colony-forming units/ml (1 McFarland suspension). Of this suspension 50 μl was added to each well to yield a final volume of 100 μl per well. After 1.5 h of incubation with shaking (900 rev./min) at 37 °C, 50 μl from selected wells was diluted 200-fold in PBS [9 mM sodium phosphate (pH 7.0)/150 mM NaCl], in which the peptides were inactive, and surviving cells were determined by plating 25 μl on SDA. After 48 h of incubation at 30 °C, the percentage viability was calculated by: (number of viable cells/mean number of viable cells in growth control) × 100%. The killing assay was also performed in 10, 20, 50 and 100 mM PPB.

Growth inhibition on agar

In addition to the killing assay, growth inhibition on agar was studied by the method of Westerhoff et al. [26]. Yeast and bacterial cells cultured for 16 h were harvested by centrifugation and diluted in PBS to a concentration to grow just confluently when plated with a cotton swab on SDA plates (yeast strains) or Schaedler F0 plates (bacterial strains). Plates were then dried for 15 min at room temperature; 5 μl (10 μg) samples of peptide were spotted on the surface of the plate. After 16 h of incubation, the plates were screened for spots of growth inhibition.

Haemolytic assay

A haemolytic assay was performed in a high-ionic-strength buffer (PBS) and in a low-ionic-strength buffer [isotonic glucose (287 mM)/1 mM PPB/2.5% PBS]. Human erythrocytes (blood group O+) were maintained in PBS and kept at 4 °C for up to 3 weeks. The erythrocytes were harvested by centrifugation and washed three times in PBS. In 96-well V-bottomed microtitre plates, peptides were diluted 2-fold serially in high-ionic-strength or low-ionic-strength buffer, starting at 167 μg/ml. Human erythrocytes were diluted in high-ionic-strength or low-ionic-strength buffer to yield a 1% suspension. Of this suspension 75 μl was added to each well (final concentration 0.5% in 150 μl total volume). After 1 h of incubation at 37 °C, the plates were centrifuged at 3000 rev./min for 5 min. The supernatant fluid was separated from the pellet and the absorbance measured at 540 nm. Non-haemolytic controls and 100% haemolysis were determined in buffer alone and in buffer containing 1% (w/v) Triton X-100 (Merck), respectively.

RESULTS

Peptide design

For the design of histatin derivatives with improved antifungal activity we synthesized substitution analogues of residues 11–24 of histatin 5 (designated dh-5), which has been reported to comprise the fungicidal domain [18]. In dh-5, projected as a regular α-helical structure of 3.6 residues per turn, both a hydrophilic and a hydrophobic face have been distinguished [19] (Figure 1). The hydrophobic face is made up of Ser^{20}, Lys^{13}, Tyr^{24}, Lys^{17}, His^{21} and Phe^{14}, the hydrophilic face of Glu^{16}, Gly^{23}, Arg^{12}, His^{19}, His^{15}, Arg^{22} and Lys^{11}, with His^{18} at the interface, resulting in a weakly amphipathic α-helix. Histidine and lysine residues at the putative hydrophobic face and possibly deprotonated histidine residues at the hydrophilic face decrease the amphipathic character of this helix. These amino acids were substituted by residues that contribute to amphipathicity. Two groups of dh-5 analogues were synthesized (Table 1). The first group consisted of peptides with substitutions at a single site, and the second group had substitutions at several sites. To eliminate the possible effect of (de)protonation, the histidine residue in the hydrophobic face was replaced by phenylalanine (analogue dh21F) and histidine residues in the hydrophilic face were replaced by lysines (dh15K, dh19K). His^{18} at the transition area was replaced by either lysine or leucine (dh18K and dh18L respectively). Lysine residues in the hydrophobic face were replaced by leucines (dh17L, dh13L), and the glycine residue in the hydrophilic face was replaced by lysine (dh23K). Histatin 5 and dh-5 were synthesized and used as reference material.

Purity analysis by reverse-phase HPLC and subsequent purification of the peptides demonstrated the screen-grade purity of one major peak with only slight contamination (less than 5%). The determined amino acid composition corresponded to the calculated values.

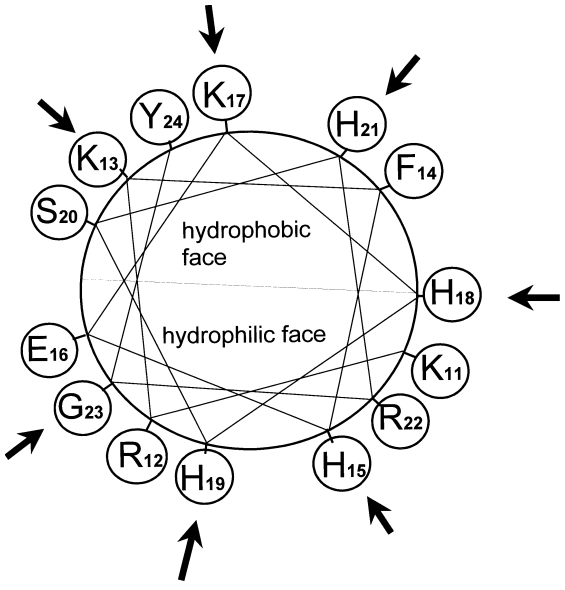

Figure 1 Helical-wheel presentation of residues 11–24 of histatin 5 (dh-5)

Substitution analogues of dh-5 are based on this diagram. The substituted residues are indicated by arrows.

Table 1 Amino acid sequences and anti-*Candida* activity of histatin analogues

Notation: +, complete inhibition; −, no inhibition; a, amide.

Peptide	Sequence	Killing IC_{50} (μM)	Growth inhibition on agar
h-5	DSHAKRHHGYKRKFHEKHHSHRGY	1.4	−
dh-5	KRKFHEKHHSHRGY	4.1	−
dh13L	KKR**L**FHEKHHSHRGY	5.2	−
dh15K	KRKF**K**EKHHSHRGY	2.1	−
dh17L	KRKFHE**L**HHSHRGY	3.0	−
dh18L	KRKFHEK**L**HSHRGY	3.0	−
dh18K	KRKFHEK**K**HSHRGY	2.6	−
dh19K	KRKFHEKH**K**SHRGY	2.5	−
dh21F	KRKFHEKHHS**F**RGY	2.9	−
dh23K	KRKFHEKHHSHR**K**Y	2.9	−
dhvar1	KR**L**F**K**E**LKF**S**L**R**K**Y**	0.6	+
dhvar2	KR**L**F**K**E**LLF**S**L**R**K**Y**	0.8	+
PGLa	GMASKAGAIAGKIAKVALKALa	0.6	+
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNSa	1.0	+
Polylysine	KKKKKKKKKKKKKKK	0.6	−
dCysSN	SSPGKPPRLVGGP	> 114	−

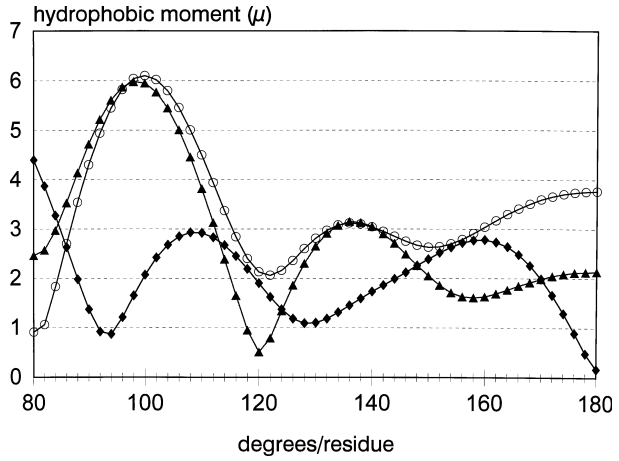

Figure 2 Hydrophobic moment profile of histatin analogues

The hydrophobic moments (μ) were plotted as a function of the angle between the amino acid side chains for dh-5 (◆), dhvar1 (○) and dhvar2 (▲).

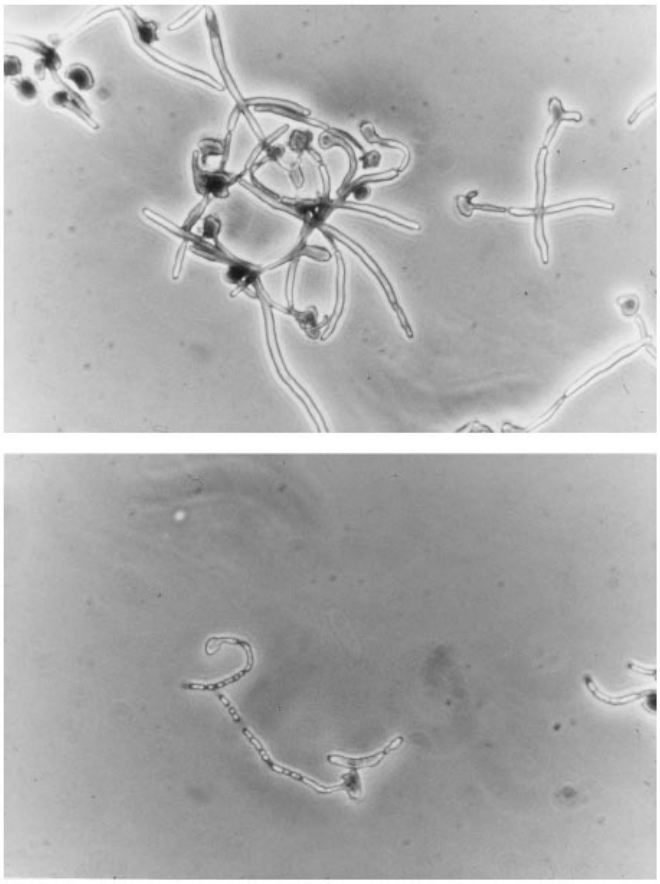

Figure 3 *C. albicans* hyphae without (upper panel) and with (lower panel) histatin 5

Photographs were taken after 1.5 h of incubation at 37 °C in 1 mM PPB without and with histatin 5 (12 μM).

In Figure 2 the hydrophobic moments (μ) of dh-5, dhvar1 and dhvar2 are given as a function of the angle (δ) between the amino acid side chains by using the equation:

$$\mu = \sum_{n=1}^{N} H_n e^{i\delta n} \quad (1)$$

where H_n is the numerical hydrophobicity of the nth residue, and N is the total number of amino acid residues [27]. The hydrophobic moment measures the amphipathicity of the N residues of a protein segment.

Analogue dh-5 displayed a maximum at 108° ($\mu = 2.9$), which indicates optimal amphipathicity in a 3_{10}-helix conformation rather than in a pure α-helix conformation, where $\delta = 100°$. The slight preference of dh-5 for a 3_{10}-helix conformation has been confirmed by CD measurements [18]. Analogues dhvar1 and dhvar2 displayed a maximum at 100°, suggesting the preference of these peptides for an α-helical conformation. Furthermore the maximum hydrophobic moment of dhvar1 and dhvar2 ($\mu = 6.1$) was about twice the maximum hydrophobic moment of dh-5.

Candidacidal activity of histatin analogues

The micrographs shown in Figure 3 show the effect of histatin 5 (12 μM) on *C. albicans* germ tubes. After 1.5 h of incubation at 37 °C, a decrease in number and size of the cells as well as fragmentation of the cell structure was observed, caused either by direct activity on the cell wall or by autolysis as a secondary effect of de-energization of the cells. No colonies were formed when the histatin-treated hyphae were plated on SDA agar. We found that the susceptibility of *C. albicans* germ tubes was of the same order of magnitude as the susceptibility of *C. albicans* blastoconidia to histatin 5 (results not shown).

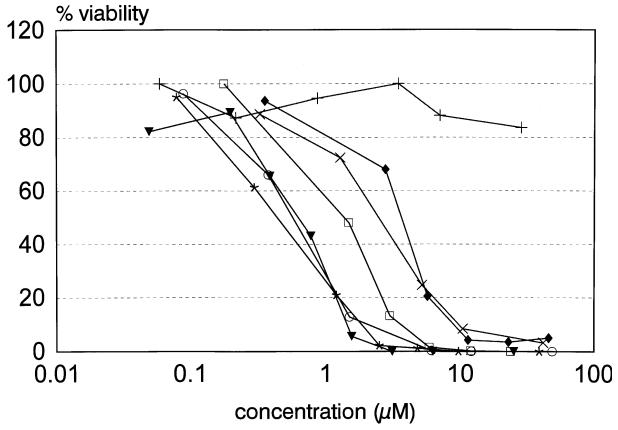

Figure 4 Anti-*Candida* activity of histatin analogues

The percentage viability was determined by plating after 1.5 h of incubation at 37 °C in 1 mM PPB with histatin 5 (□), dh-5 (◆), dh19K (×), dhvar1 (○), PGLa (★), polylysine (▼) and dCysSN (+, negative control).

In Figure 4 the killing curves are shown for dh-5, dh19K (containing a single substitution) and dhvar 1 (containing several substitutions). PGLa, a helix-forming broad-spectrum antimicrobial peptide of amphibian origin, was used for comparison, and 15-mer polylysine was tested as a positively charged control. As a negative control a synthetic peptide was included that encompassed residues 1–14 of cystatin SN (dCysSN), which displays neither helix formation nor amphipathicity.

From these killing curves the IC_{50} values (concentrations at which half the *Candida* inoculum was killed) were determined (Table 1). All killing experiments were performed in triplicate on different occasions, and the mean IC_{50} values are shown. The variation in IC_{50} values between experiments was 30% at most.

The activity of dh-5 was found to be one-third that of the complete synthetic histatin 5 molecule (IC_{50} values 4.1 and 1.4 μM respectively). The IC_{50} values of dh-5 analogues with single-site substitutions ranged from 2.1 to 5.2 μM, indicating that substitutions at single sites had little effect on the fungicidal potency of dh-5. Analogues with multi-site substitutions were more fungicidal than the reference peptides: the IC_{50} values of dhvar1 and dhvar2 were 0.6 and 0.8 μM respectively, a 6-fold increase over dh-5 and a 2-fold increase over histatin 5. The activity of dhvar1 and dhvar2 was comparable to the activity of PGLa and magainin 2. Remarkably, under these test conditions polylysine was fungicidal to the same extent as dhvar1 and PGLa (IC_{50} 1 μM).

The presence of ions can affect the activity of several antimicrobial peptides, including histatins [14]. The activities of histatin 5, dhvar1, dhvar2, PGLa and magainin 2 were therefore investigated at increasing PPB concentrations (1, 10, 20, 50 and 100 mM). Figure 5 shows that the activities of all peptides decreased at increasing PPB concentration. Histatin 5 was most sensitive: in buffer concentrations above 20 mM PPB, little or no

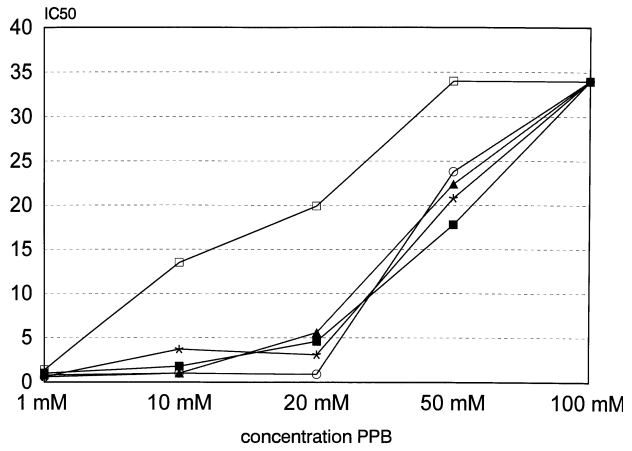

Figure 5 Influence of the ionic strength on candidacidal activity

The IC_{50} values of histatin 5 (□), dhvar1 (○), dhvar2 (▲), PGLa (★) and magainin 2 (■) are shown at increasing PPB concentration. The highest peptide concentration tested was 32 μM.

Table 2 Growth inhibition on agar

Notation: +, complete inhibition; +/−, partial inhibition; −, no inhibition.

	Growth inhibition					
Micro-organism	Histatin 5	dhvar1	dhvar2	PGLa	Magainin 2	Polylysine
C. albicans	−	+	+	+	+	−
T. glabrata	−	+	+	+	+	−
S. mutans HG 982	−	+	+	+	+	−
S. mutans HG 1002	−	+	+	+	+	−
S. mutans Ingbritt	−	+	+	+	+	−
S. sanguis	−	+	+	+	+	−
A. actinomycetemcomitans	−	−	−	+/−	+	−
F. nucleatum	−	+	+	+	+	−
V. parvula	−	+	+	+	+	−
P. intermedia	−	+	+	+	+	−
Staph. aureus (MRSA)	−	+/−	+/−	+	+	−

fungicidal activity was observed. In contrast, dhvar1, dhvar2, PGLa and magainin 2 were still active at 20 mM PPB, but IC_{50} values increased at buffer concentrations above 50 mM PPB. In 100 mM PPB, less than 50% killing was found at the highest peptide concentrations tested (32 μM). The same effect was observed at increasing concentrations of NaCl (results not shown).

Growth inhibition on agar

Growth inhibition on agar (visible as a circular clear spot after 16 h of incubation) was scored as positive when observed at least twice in three independent experiments (Table 1). Histatin 5, dh-5, singly substituted dh-5 analogues and polylysine did not inhibit the growth of *C. albicans* in this assay. In contrast with these peptides, dhvar1, dhvar2, PGLa and magainin 2 caused clear spots of growth inhibition.

To investigate whether dhvar1 and dhvar2 exhibited broad-spectrum activity, the susceptibility of a number of oral and non-oral pathogenic micro-organisms was tested in the same way (Table 2). *T. glabrata*, comprising 10% of the clinical isolates [28], three strains of *S. mutans*, associated with caries, *S. sanguis*, an oral commensal, and *F. nucleatum*, *V. parvula* and *P. intermedia*, associated with periodontitis, were inhibited by dhvar1 and dhvar2. The growth of a methicillin-resistant *Staph. aureus* strain (MRSA) was partly inhibited; some surviving cells grew in the clear spot (indicated by +/−). The growth of *A. actinomycetemcomitans*, a species that is related to juvenile periodontitis, was not inhibited by dhvar1 and dhvar2. Magainin 2 inhibited all micro-organisms tested, including *A. actinomycetemcomitans*. In this assay, neither histatin 5 nor polylysine inhibited any of these oral micro-organisms. When histatin 5 was spotted at a concentration 10 times higher (100 μg), the growth of *C. albicans* and *S. sanguis* was slightly inhibited (results not shown).

Table 3 Fungicidal and haemolytic activities

Abbreviations: LI, low-ionic-strength; HI, high-ionic-strength.

	IC_{50} (μM)		Concentration for 50% haemolysis (μM)	
Peptide	In LI buffer	In HI buffer	In LI buffer	In HI buffer
Histatin 5	2.9	> 23	> 32	> 32
dhvar1	0.9	> 48	5.3	> 65
dhvar2	0.9	> 42	7.1	> 57
PGLa	4.9	> 39	10.3	> 52
Magainin 2	2.3	> 29	2.9	> 39
Polylysine	1.3	> 25	> 33	> 33

Fungicidal and haemolytic activities

For a proper comparison of the fungicidal and haemolytic activities, both assays were conducted in the same buffers. Because it was found that the fungicidal activity was strongly dependent on the ionic strength, the fungicidal and haemolytic activities of a number of peptides were determined at high- and low-ionic-strength isotonic conditions (Table 3). The haemolytic assay was performed three times on independent occasions. The variation between experiments was 35% at most. The fungicidal activity was determined by the standard viability assay. In low-ionic-strength buffer all peptides displayed fungicidal activities, with IC_{50} values ranging from 0.9 to 4.9 μM. Those peptides exhibiting broad-spectrum antimicrobial activity (dhvar1, dhvar2, PGLa and magainin 2) also showed haemolytic activity (50% haemolysis at 5.3, 7.1, 10.3 and 2.9 μM respectively). In contrast, histatin 5 and polylysine were not haemolytic under these conditions at concentrations up to 32 μM. Under high-ionic-strength conditions (PBS), the peptides were not active, either against fungal cells or against erythrocytes.

DISCUSSION

In the present study we investigated the effect of substitutions in a histatin derivative, dh-5, on antimicrobial and haemolytic activities, thereby focusing on helix amphipathicity. One of the key determining features of a peptide to act as a membrane-active antimicrobial peptide is the ability to adopt helical conformations with lateral amphipathicity [8,29]. These properties are linked because sequences that form α-helices tend to have a strong periodicity in the hydrophobicity of 3.6 residues, the

period of the α-helix; in contrast, amphiphilicity is a factor in the folding of proteins [20,22,27]. Because of their basic nature and weakly amphipathic character, histatins are considered to be members of the family of membrane-active antimicrobial peptides [8,19]. A reasonable approach to improving the antimicrobial activity of dh-5 was to replace those amino acid residues that do not contribute to the spatial segregation of hydrophobic and hydrophilic amino acids (hydrophobic moment) in an optimal α-helix model. In this model, substitutions at single loci in dh-5 do not significantly improve the α-helix amphipathicity (hydrophobic moment of dh-5, 2.07; mean hydrophobic moment of the single-site substitution analogues, 2.67) and we found no significant effect on the antifungal activity. However, the amphipathicity of dhvar1 and dhvar2 (mean hydrophobic moment, 6.03) was increased 3-fold when compared with dh-5 in an α-helix conformation, or 2-fold when compared with dh-5 in a 3_{10}-helix conformation. In accordance with these data, a significant increase in antifungal activity was observed. Moreover, broad-spectrum activity emerged as a result of these substitutions and in terms of their antifungal and haemolytic activities a comparison between dhvars and magainins seems to be allowed.

We found that substitution of amino acid residues at positions 13, 15, 17, 18, 19, 21 and 23 of dh-5 hardly influenced antifungal activity. It is worth noticing that in dhvar1 and dhvar2 all histidine residues had been replaced. Therefore the high histidine content in both the antifungal protein from *Sarcophaga peregrina* and histatins does not seem to be an explanation for their shared antifungal activity [30]. Our findings are consistent with the reports on magainins and cecropins that amphipathic structures rather than strict primary sequences are important for membrane permeation, and that specific peptide/receptor interactions are not likely to be involved [8].

Besides the ability to form amphipathic structures, a net positive charge is essential for activity [8]. In our killing assay, polylysine was as active as a fungicide as PGLa, dhvar1 and dhvar2. The mechanism of antibacterial activity of short polylysine peptides (10–30-mer) is generally ascribed to electrostatic adsorption to the cell surface, followed by stripping of the outer membrane and abnormal distribution of the cytoplasm [31]. Comparable effects were observed for *Candida* on incubation with histatin 5. Because all basic peptides tested exhibited antifungal activity in low-ionic-strength buffer, it cannot be excluded that, under these conditions, the mechanism of fungicidal activity of histatins is comparable to that of polylysine, namely membrane disintegration as a result of peptide adsorption to negatively charged groups on the membrane. This is further confirmed by the finding that under less favourable test conditions (e.g. at higher ionic strength) polylysine and histatin 5 show a strong decrease in fungicidal potency, suggesting that their activity relies mainly on weak electrostatic interactions. The stronger activity of the analogues dhvar1 and dhvar2 compared with histatin 5 and polylysine was demonstrated at conditions of higher ionic strength and by the spot-assay on agar.

The involvement of electrostatic interactions has been demonstrated for a broad range of basic antimicrobial peptides, including histatins, by a decrease in activity at increasing ionic strength [5,14,30,32–35]. This phenomenon is generally explained by the shielding of charged groups on the peptide and the membrane, thereby preventing interaction between them [36]. We found an ionic strength dependence for all histatin analogues. However, dhvar1 and dhvar2, unlike histatin 5, were still highly active at 20 mM PPB, for which the ionic strength is comparable to that of saliva. The difference in the activities of histatin 5 on the one hand and the broad-spectrum peptides on the other hand might reflect the difference in the strength of the binding and hence in the affinity as determined by hydrophilic and hydrophobic interactions and by the proper peptide conformation.

It is thought that antimicrobial peptides are preferentially selective for prokaryotic membranes, but the basis for specificity at a molecular level is not yet known. Liposomes composed of anionic phospholipids are efficiently lysed by cecropins [37] and by magainins [38]. The absence of acidic phospholipids from the outer leaflet of erythrocytes and the abundant presence of cholesterol combined with a weak transmembrane potential contribute to the protection of erythrocytes against attack by magainins. The generation of an inside negative potential enhances magainin-induced haemolysis [36,38]. These reports indicate that electrostatic interactions are necessary for effective lysis. Testing the haemolytic activity of antimicrobial peptides is usually performed in PBS [22,39,40,41]. We found no haemolytic activity on testing in PBS, but also no antifungal activity of histatin derivatives and magainins. At lower ionic strength, the most active peptides (dhvars and magainins) caused haemolysis, so that the apparent specificity of this kind of peptides for the microbial membrane is nullified at low ionic strength. Thus the present study suggests that enhanced antimicrobial activity is concomitant with increased haemolytic activity. Improvement of the antimicrobial activity of magainin 2 increased its ability to lyse erythrocytes [21]. However, other studies on model amphipathic peptides report that enhancement of antimicrobial activity is not necessarily coupled with an increase in haemolytic activity [20,42]. In contrast with the latter studies, we performed the fungicidal and the haemolytic assay in the same buffers, which makes the results fully comparable.

This study demonstrates that the α-helix model of dh-5 is a good starting point for developing histatin analogues with improved antimicrobial activity. It seems that modifications that enhance amphipathicity are attended with the appearance of broad-spectrum activity and hence the loss in specificity. Although this model seems applicable, the mechanism by which the analogues kill their target organism might be different from that of dh-5 or natural histatin 5.

Because of the increasing number of microbial strains with intrinsic resistance or decreased susceptibility emerging during prophylaxis or therapy, there is a pressing need for the development of new antimicrobial agents with broad-spectrum antimicrobial activity [43]. Broad-spectrum antimicrobial peptides interacting with essential cell wall components, such as lipopolysaccharides or acidic phospholipids, are expected to be less vulnerable to resistance and might find an application in the treatment of infections caused by persistent micro-organisms. For example, the efficacy of a magainin analogue is currently being evaluated in clinical trials for the treatment of impetigo and diabetic ulcers [8]. Until now, no induction of resistance against these kind of peptide has been described. Histatins are mammalian oral anticandidal peptides, and analogues with improved activity might provide a good alternative or supplement to prevent the recurrence of oral candidiasis.

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