A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides

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Abstract The hemolytic and fungicidal activity of a number of cationic antimicrobial peptides was investigated. Histatins and magainins were inactive against human erythrocytes and *Candida albicans* cells in phosphate buffered saline, but displayed strong activity against both cell types when tested in 1 mM potassium phosphate buffer supplemented with 287 mM glucose. The HC_{50}/IC_{50} ratio, indicative of the therapeutic index, was about 30 for all peptides tested. PGLa was most hemolytic ($HC_{50} = 0.6$ μM) and had the lowest therapeutic index ($HC_{50}/IC_{50} = 0.5$). Susceptibility to hemolysis was shown to increase with storage duration of the erythrocytes and also significant differences were found between blood collected from different individuals. In this report, a sensitive assay is proposed for the testing of the hemolytic activities of cationic peptides. This assay detects subtle differences between peptides and allows the comparison between the hemolytic and fungicidal potency of cationic peptides.

Key words: Hemolytic activity; Fungicidal activity; Cationic peptide; Antimicrobial

1. Introduction

Throughout the plant and animal kingdom numerous cationic peptide antibiotics, such as cecropins [1], defensins [2], magainins [3] and histatins [4–6] have been discovered which provide the host with a non-immune first-line defense system against invading microorganisms. Because of their strong growth-inhibitory and microbicidal activity against pathogenic bacteria and yeasts in vitro, these peptides have often been used as design templates for the development of anti-infectious therapeutics [7–10]. To improve the therapeutic index of these compounds, antimicrobial activity with minimal cytotoxicity towards host cells is needed. As an indication for this index, the lytic activity against human erythrocytes is often compared to the antibacterial activity in growth inhibition assays [11–15]. Based on this comparison it is claimed that natural magainins, mucosal β-defensins, and cecropins react selectively with the microbial membrane, because they are not hemolytic [16–19]. In contrast to the activity of bee venom melittin, which is often used as a positive control peptide in the hemolytic assays [15,20], the activities of many cationic peptides is strongly dependent on the ionic strength [9,21–23]. As isotonic conditions are required to prevent erythrocytes from spontaneous lysis, hemolytic assays are usually performed in Tris or phosphate buffered saline (PBS) [11–15], which for antimicrobial assays are considered to be high salt buffers. Therefore, it is questionable whether the results of hemolytic assays that are conducted in such buffers can be compared properly with the results of antibacterial assays that are conducted in low ionic strength buffers or broths.

In the present study, the hemolytic and candidacidal activities of cationic peptides from human and amphibian origin (histatins and magainins, respectively) and of a number of designed analogs, derived from histatins, were investigated in high and low ionic strength buffers. The peptides tested differ in chain length, net peptide charge, hydrophobicity and amphipathicity, thus allowing the effect of these parameters on peptide activity and selectivity to be investigated. In Table 1, the sequences of the chemically synthesized peptides used in this study are listed. Histatin 5 is a human salivary peptide, dhvar1–dhvar5 are analogs of residues 11–24, the fungicidal domain of histatin 5, located at its C-terminus. Dhvar1 and dhvar2 were designed to exhibit improved amphipathicity in an α-helix [9], dhvar3 and dhvar4 are derived from dhvar1 and dhvar2, respectively, by substitution of the negatively charged glutamic acid (E) by a lysine (K), which yielded a net increase in positive charge of two units. The sequestration of hydrophobic and hydrophilic amino acids perpendicular to the axis of the α-helix was quantified by calculating the mean hydrophobic moment per residue (μ), with the angle (δ) between amino acid side chains set at 100° [24]. According to this calculation, histatin 5 is only weakly amphipathic having a μ of 0.091. Dhvar1–dhvar4, PGLa, and magainin 2 have mean hydrophobic moments per residue ranging from 0.260 to 0.441 demonstrating the amphipathic character of these peptides. Dhvar5 was designed to have a minimal μ perpendicular to the helix ($\mu = 0.064$). In contrast to the other peptides, dhvar5 can adopt a segmentally amphipathic α-helix with a hydrophobic N-terminus and a hydrophilic C-terminus. The mean hydrophobicity per residue was calculated using the consensus scale for hydrophobicity [24]. All peptides were positively charged at neutral pH, except dcysSA, the N-terminal 14 residue sequence of cystatin SA, which was used as a negative control peptide. At neutral pH, and upon interaction with the lipophilic inside of the phospholipid bilayer, the histidine side chains may be either positively charged or uncharged, which dramatically changes the charge of histatin 5 as it contains seven histidine residues.

It was found that at low ionic strength, in contrast to in PBS, subtle differences in activity between peptides are detectable. Using this sensitive assay, the hemolytic and the fungi-

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cidal properties of cationic peptides have been compared as a more accurate measure for their therapeutic index.

2. Materials and methods

Peptides were chemically synthesized as described previously [25] using the T-bag method adapted for 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. In brief, *p*-benzyloxybenzyl alcohol resins, with the first *N*-Fmoc-protected amino acids already attached, were included inside the T-bags. The coupling reactions were performed in *N*,*N*-dimethylformamide. After completion of the sequence, cleavage from the resin and simultaneous side chain deprotection was achieved with a mixture of 5% thioanisole, 5% phenol, 5% water and 85% trifluoroacetic acid. Purity analysis was performed by reversed phase HPLC, and revealed one major peak with only slight contaminations ($<5\%$). Chemically synthesized PGLa and magainin 2 were kindly provided by H.V. Westerhoff (Department of Microbial Physiology, Vrije Universiteit, Amsterdam, The Netherlands) and B.J. Appelmelk (Department of Medical Microbiology, Vrije Universiteit, Amsterdam, The Netherlands), respectively. All peptides were dissolved in 10 mM PPB (potassium phosphate buffer, pH 7.0) to 2 mg/ml and stored at −20°C. The final pH of the stock solution was 6.0.

Human erythrocytes from four healthy individuals were collected in vacuum tubes containing heparin (final concentration 20.4 U/ml) or citrate (final concentration 0.129 M) as anti-coagulant. The study was approved by the medical ethics committee of the Vrije Universiteit, and informed consent was obtained from all individuals. The erythrocytes were harvested by centrifugation for 10 min at $2000\times g$ at +20°C, and washed three times in PBS (9 mM sodium phosphate, pH 7.0 in 150 mM NaCl). To the pellet PBS was added to yield a 20% (v/v) erythrocytes/PBS suspension. This cell suspension was used either directly or stored for 3 weeks at 4°C. In the latter case the cells were washed once in PBS to clear the supernatant from hemoglobin released from lysed erythrocytes during the period of storage. The 20% suspension was diluted 1:20 in PBS or in IGP (isotonic glucose phosphate buffer, 1 mM PPB, pH 7.0, supplemented with 287 mM glucose as an osmoprotectant) and from this suspension 100 μl was added in duplicate to 100 μl of a two-fold serial dilution series of peptide in the same buffer in a 96-well V-bottomed microtiter plate (Greiner, Recklinghausen, Germany). Total hemolysis was achieved with 1% Tween-20. The plates were incubated for 1 h at 37°C and then centrifuged for 5 min at $1500\times g$ at +20°C. Of the supernatant fluid 150 μl was transferred to a flat-bottom low affinity microtiter plate (Greiner), and the absorbance was measured at 450 nm. The percentage hemolysis was calculated by: [(A_{450} of the peptide treated sample−A_{450} of buffer treated sample)/(A_{450} of Tween-20 treated sample−A_{450} of buffer treated sample)]×100%, and the HC_{50} values, which represent the concentrations of peptide at which 50% hemolysis was observed, were determined.

Candida albicans (ATCC 10231) was stored on glycerol at −80°C and cultured on Sabouraud dextrose agar (Oxoid, Hampshire, UK) for 48 h at 30°C. Several colonies were picked from plate and suspended in PBS or IGP buffer to 2×10^7 colony forming units (CFU)/ml. Of this suspension 100 μl was added in duplicate to 100 μl of a two-fold serial dilution series of peptide in the same buffer in a V-bottom microtiter plate (Greiner). After 1 h of incubation, 50 μl from selected wells was diluted in 9 ml of PBS and 25 μl was plated on Sabouraud dextrose agar. After 48 h of incubation at 30°C, the percentage killing was determined by: [1−(number of viable counts of the peptide treated sample/mean number of viable counts in three buffer treated samples)]×100%, and the IC_{50} values, which represent the concentrations of peptide at which 50% of the yeast inoculum was killed, were determined.

Statistical analyses were performed using the SPPS/PC+ Statistical Software Package, version 6.0 for Windows (SPPS Inc., Chicago, IL, USA). The effects of storage duration and anti-coagulant on hemolysis (Table 4) were both analyzed by one-way ANOVA with 'peptide treatment' as covariant. Comparison between the susceptibility of erythrocytes from different individuals or between the hemolytic activity of different peptides (Table 3) was carried out by a one-way ANOVA, with 'peptide treatment' or 'individual' as covariant, respectively, followed by Wilcoxon matched-pairs signed rank tests when appropriate. Levels of significance were set at $P<0.05$.

3. Results and discussion

The hemolytic activity of membrane-active antimicrobial peptides against human erythrocytes is often used as a measure for their cytotoxicity and to estimate their therapeutic index [11–15]. Based on the absence or the presence of hemolytic activity, these peptides are classified as peptide antibiotics or peptide venoms, respectively [16]. In the present study we show that the hemolytic properties of cationic peptides are strongly dependent on the conditions under which the hemolytic assay is performed, such as the ionic strength of the incubation buffer, the freshness of the erythrocytes and even on individual differences in blood group antigens.

The killing activities of histatins and magainins against *C. albicans*, measured by the loss in CFU/ml by plating on agar, were negligible in PBS, which is considered a high ionic strength buffer in candidacidal assays. Only at the highest test concentrations of dhvar3, dhvar4 and dhvar5 the viable counts were reduced by 10%, 25% and 90%, respectively (Fig. 1). Likewise, no hemolytic activity against human erythrocytes, measured by hemoglobin release, was observed, not even at the highest peptide concentrations. In contrast, when the experiments were conducted in IGP, all peptides were both fungicidal and hemolytic, the negative control peptide dcysSA excepted (Fig. 2). In buffer containing sorbitol instead of glucose, similar results were obtained (data not shown).

Table 1
Some physical characteristics of the peptides used in this study

Peptide	Amino acid sequence	μ[a]	H[b]	Net charge at neutral pH[c]
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	0.091	−0.547	5+/12+[d]
Dhvar1	KRLFKELKFSLRKY	0.436	−0.426	5+
Dhvar2	KRLFKELLFSLRKY	0.425	−0.310	4+
Dhvar3	KRLFKKLKFSLRKY	0.441	−0.461	7+
Dhvar4	KRLFKKLLFSLRKY	0.439	−0.344	6+
Dhvar5	LLLFLLKKRKKRKY	0.064	−0.410	7+
PGLa	GMASKAGAIAGKIAKVALKAL-a	0.260	+0.042	5+
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	0.286	−0.036	3+/4+[d]
DcysSA	WSPQEEDRIIEGGI	0.214	−0.176	3−

[a] μ: mean hydrophobic moment per residue of the peptide in α-helical conformation [24].
[b] H: mean hydrophobicity calculated from the sum of hydrophobicity values [24].
[c] The net charge was calculated by the number of (K+R)−(E+D). In PGLa, the positive charge at the N-terminus was not compensated by a negative charge at the C-terminus, due to amidation, thus increasing the net peptide charge by 1 unit.
[d] Depending on the charge of histidine ($pK_a = 6.0$).

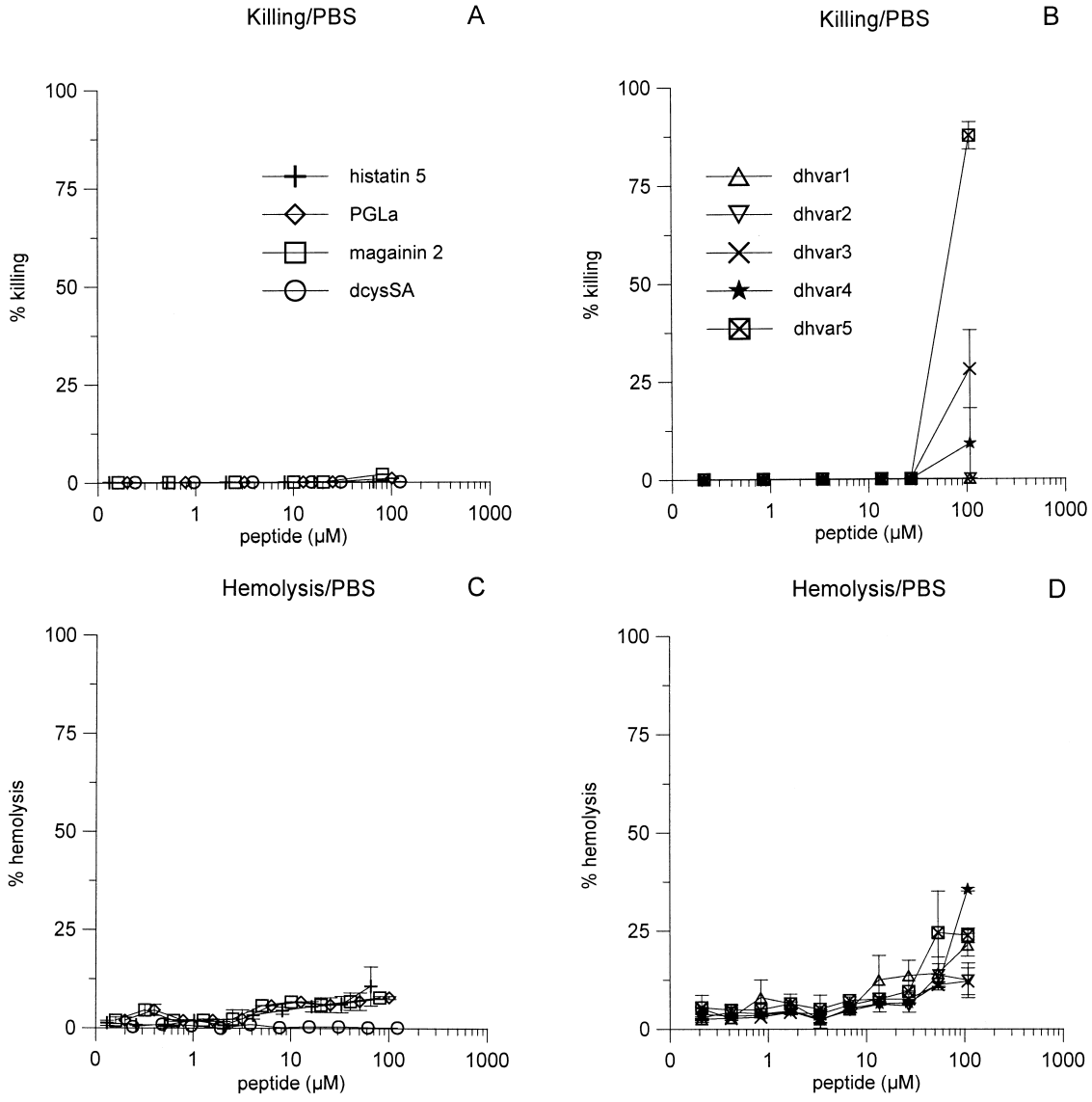

Fig. 1. Fungicidal activities (A for naturally occurring peptides and control, B for designed variants) and hemolytic activities (C for naturally occurring peptides and control, D for designed variants) in PBS. *C. albicans* cells (10^7 CFU/ml) were incubated for 1 h at 37°C with a two-fold serial dilution series of peptide. Viable counts were determined by plating on Sabouraud agar. Human erythrocytes (final concentration 0.5%) were incubated for 1 h at 37°C with a two-fold dilution series of peptide. Hemolysis was determined by measurement of the OD_{450} of the supernatant and compared to hemolysis achieved with 1% Tween-20. Values represent the mean of duplicate experiments ± S.D. ($n = 2$).

These results show that a series of peptides, including magainins and histatins, that are generally classified as peptide antibiotics based on the absence of hemolytic activity in PBS, do display hemolytic activity when tested under low ionic strength conditions. Based on the absence of hemolysis in PBS, it has been suggested that the abundance of negatively charged sialic acids on the erythrocytes, which are about 80 Å away from the red blood cell surface, would protect the cells against lysis by neutralizing the peptides [18]. We show here that this argument does not apply when the sensitivity of the assay is increased. With respect to ionic strength dependence, it has been reported that even the hemolytic activity of melittin, which is hemolytic in PBS and is classified as a peptide venom, is inhibited when the phosphate concentration is raised to 500 mM [26]. This and our findings suggest that the peptide-erythrocyte interaction is governed by electrostatic interactions.

The hemolytic activity of cationic peptides in PBS is often compared to the antibacterial activity, measured as growth inhibition in low ionic strength broth, e.g. Müller-Hinton broth [27]. Besides the fact that the incubation buffers and the incubation times differ, the two assays measure different properties, namely the membrane perturbing and growth inhibitory activity, respectively, which hampers a proper comparison between the outcomes. In the present study, the fungicidal and hemolytic activities are compared after the same incubation time and in the same buffer. Assuming that both killing and hemolysis are the final consequence of peptide binding, we expressed the selectivity of the peptides for the different cell types as the ratio of the HC_{50} and IC_{50} values (Table 2). For all cationic peptides, except for PGLa, the HC_{50} values were higher than the IC_{50} values. The mean HC_{50}/IC_{50} of the remaining seven peptides was 34 ± 15, indicating that histatins and magainin 2, but not PGLa, express

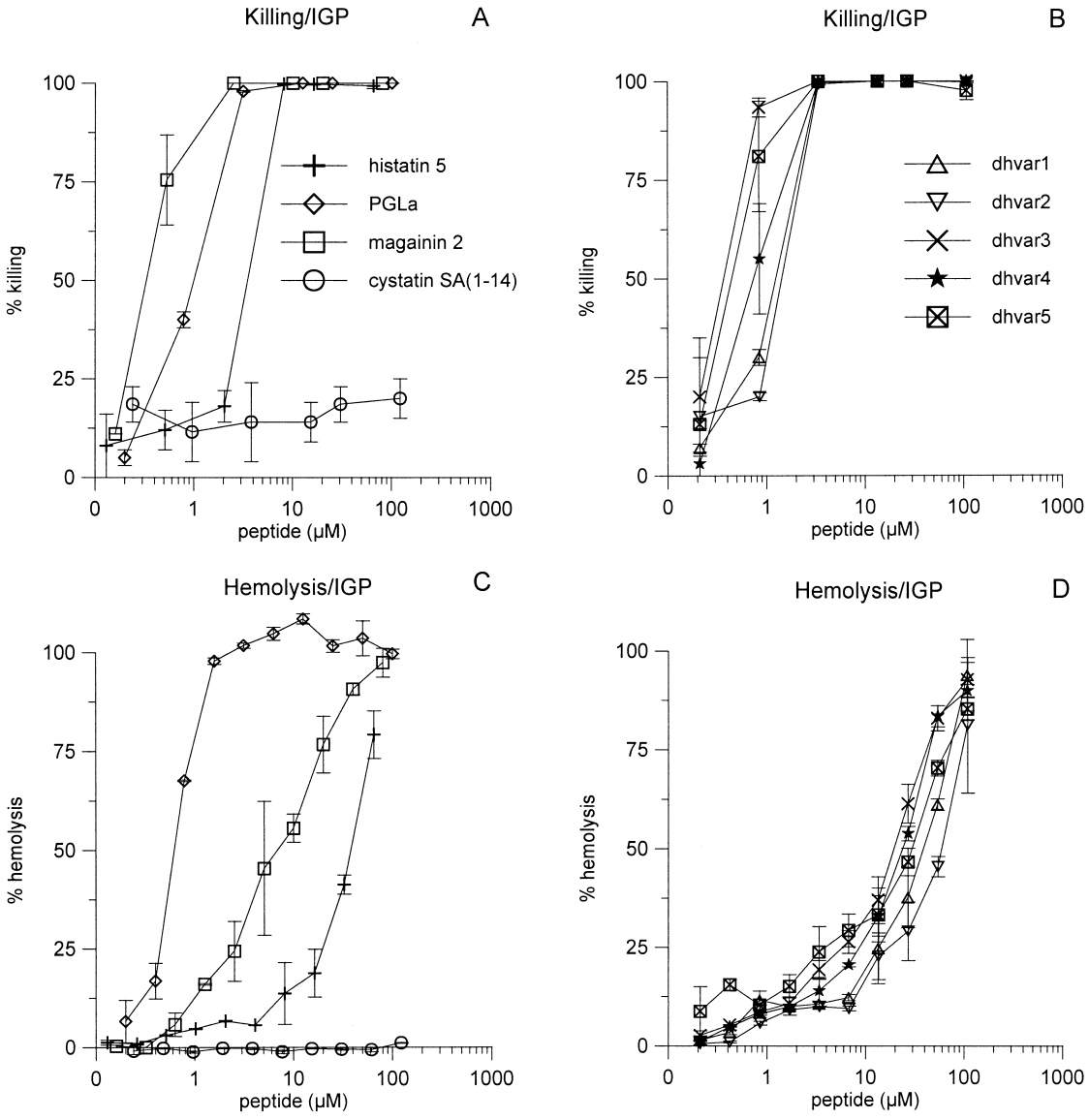

Fig. 2. Fungicidal activities (A for naturally occurring peptides and control, B for designed variants) and hemolytic activities (C for naturally occurring peptides and control, D for designed variants) in IGP buffer. See the legend of Fig. 1 for experimental details.

Table 2
Fungicidal and hemolytic activities of cationic peptides in low and high ionic strength buffers

	In IGP[a]			In PBS[b]		
	IC_{50} (μM)[c]	HC_{50} (μM)[d]	HC_{50}/IC_{50}	IC_{50} (μM)	HC_{50} (μM)	HC_{50}/IC_{50}
Histatin 5	2.5 ± 1.5[e]	39 ± 1.5	16	> 66	> 66	n.d.[f]
Dhvar1	1.3 ± 0.1	36 ± 4.0	28	> 108	> 108	n.d.
Dhvar2	1.6 ± 0.1	59 ± 0.1	37	> 109	> 109	n.d.
Dhvar3	0.4 ± 0.1	20 ± 2.5	50	> 108	> 108	n.d.
Dhvar4	0.9 ± 0.3	23 ± 2.0	26	> 109	> 109	n.d.
Dhvar5	0.5 ± 0.1	29 ± 2.5	58	50 ± 4.1	> 108	n.d.
PGLa	1.1 ± 0.1	0.6 ± 0.1	0.5	> 102	> 102	n.d.
Magainin 2	0.3 ± 0.1	7.2 ± 3.4	24	> 81	> 81	n.d.
DcysSA	> 123	> 123	n.d.	> 123	> 123	n.d.

[a]IGP (low ionic strength buffer): 1 mM potassium phosphate, pH 7.0, supplemented with 287 mM glucose.
[b]PBS (high ionic strength buffer): 9 mM sodium phosphate buffer, pH 7.0, supplemented with 150 mM NaCl.
[c]IC_{50}: concentration of peptide which gives 50% reduction in viable counts of *C. albicans* cells compared to buffer treatment.
[d]HC_{50}: concentration of peptide which gives 50% hemolysis of human erythrocytes compared to Tween-20 treatment.
[e]Values represent the mean of duplicate experiments ± S.D. ($n = 2$).
[f]n.d.: not calculated.

Table 3
Influence of anti-coagulant and storage duration of erythrocytes on the susceptibility to lysis by cationic antimicrobial peptides

Peptide	HC_{50} (μM)[a]			
	Heparin blood[b]		Citrate blood[c]	
	$t = 0$ days	$t = 21$ days	$t = 0$ days	$t = 21$ days
Histatin 5	39 ± 1.5	9.0 ± 1.0	38 ± 2.0	15 ± 4.0
Dhvar1	36 ± 4.0	5.6 ± 0.6	51 ± 9.0	4.3 ± 0.8
Dhvar2	59 ± 0.1	2.8 ± 0.8	59 ± 1.0	5.7 ± 0.3
Dhvar3	20 ± 2.5	2.4 ± 0.4	25 ± 2.0	3.0 ± 0.1
Dhvar4	23 ± 2.0	3.4 ± 0.6	30 ± 2.0	3.0 ± 0.1
Dhvar5	29 ± 2.5	1.7 ± 0.2	38 ± 2.0	1.5 ± 0.1
PGLa	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
Magainin 2	7.2 ± 3.4	2.2 ± 0.8	7.5 ± 0.5	0.5 ± 0.1
DcysSA	> 123	68 ± 7.5	> 123	98 ± 23

[a]Concentration of peptide at which 50% hemolysis compared to Tween-20 treatment was observed. Values represent the mean of duplicate experiments ± S.D. ($n = 2$).
[b]Blood collected in heparin-containing tubes.
[c]Blood collected in citrate-containing tubes.

selectivity for the fungal cell over the erythrocyte. In the case of PGLa the poor selectivity is the result of a high activity against the erythrocytes rather than a low activity against the fungal cell.

When the peptides were ranked according to their hemolytic activities, the following order was found: PGLa > magainin 2 > all histatin variants. Interestingly, PGLa has the highest mean hydrophobicity value (+0.042), followed by magainin 2 (−0.036), followed by the histatins (−0.310 to −0.547). No correlation was found between the hemolytic activity and mean amphipathicity per residue (μ) or the net peptide charge. This is in agreement with studies of Kiyota et al. [28] and Mor et al. [29] who found a positive correlation between the hydropathic index of peptides and their hemolytic potential. Also, the results of structure-activity studies on model peptides derived from magainins show that with an increase in peptide hydrophobicity the degree of specificity decreases [19]. It is believed that although electrostatic interactions determine the initial attraction, membranes composed of zwitterionic phospholipids such as the outer leaflet of the erythrocyte would favor hydrophobic interactions allowing a deeper insertion and a stronger interaction of the peptide with lipophilic membrane components leading to efficient lysis [30].

To assess the influence of anti-coagulant and storage duration on the susceptibility of human erythrocytes to lysis, blood was collected in tubes containing either heparin or citrate as the anti-coagulant, and tested both directly and after 3 weeks of storage at 4°C (Table 3). Statistical analysis of the data revealed that there are no significant differences between heparin and citrate blood tested either after 0 or after 21 days of storage at 4°C. In contrast, storage itself caused a significant increase in the susceptibility to hemolysis, as shown by the decrease in HC_{50} values ($P < 0.0005$ for both heparin and citrate blood). Only PGLa was equally active against fresh erythrocytes and erythrocytes stored for 3 weeks (mean HC_{50} value 0.6 ± 0.1 μM). The mean ratio [HC_{50} (erythrocytes tested at $t = 0$)/HC_{50} (erythrocytes tested at $t = 21$ days)] ± S.D. for the remaining seven cationic peptides was 9.7 ± 6.6 for heparin blood and 10.9 ± 6.9 for citrate blood, indicating that storage for 3 weeks at 4°C increases the susceptibility of red blood cells to hemolysis approximately 10-fold. This might be explained by a time-dependent decrease of ATP levels in the erythrocyte. Hemolysis by membrane active peptides is most likely the result of perturbation of the lipid bilayer followed by colloid osmotic lysis [26]. It is conceivable that fresh erythrocytes, having the ability to regulate their volume by protein pumps, would be able to counteract small disruptions in the cellular membrane [31]. This energy-dependent restoration mechanism might be lost upon long-term storage.

To assess whether individual differences in antigen expression on red blood cells influence the susceptibility to hemol-

Table 4
Influence of individual differences in the susceptibility of freshly collected erythrocytes to hemolysis by cationic antimicrobial peptides

Peptide	HC_{50} (μM)[a]				
	Individual 1	Individual 2	Individual 3	Individual 4	
Histatin 5	49 ± 9.0	> 66	36 ± 4.0	39 ± 1.5	X
Dhvar1	34 ± 24	89 ± 20	58 ± 8.0	36 ± 4.0	0.463
Dhvar2	89 ± 12	> 108	73 ± 2.0	59 ± 0.1	0.012[b]
Dhvar3	51 ± 21	83 ± 13	35 ± 3.0	20 ± 2.5	0.866
Dhvar4	42 ± 12	58 ± 2.0	35 ± 3.0	23 ± 2.0	0.018[c]
Dhvar5	58 ± 23	99 ± 10	65 ± 15	29 ± 2.5	0.141
PGLa	0.6 ± 01	0.6 ± 01	0.6 ± 0.1	0.6 ± 0.1	0.012[c]
Magainin 2	19 ± 1.0	17 ± 1.0	8.0 ± 0.1	7.2 ± 3.4	0.012[c]
DcysSA	> 123	> 123	> 123	> 123	n.d.[d]

[a]Concentration of peptide at which 50% hemolysis compared to Tween-20 treatment was observed. Values represent the mean of duplicate experiments ± S.D. ($n = 2$). Individuals 1–4 express blood groups A, B, AB and O antigens, respectively. Right-hand column: analysis of variance (P values) of the HC_{50} values of a number of peptides against human erythrocytes.
[b]HC_{50} value of the peptide is significantly higher than that of histatin 5.
[c]HC_{50} values of the peptide is significantly lower that of histatin 5.
[d]n.d.: not done.

ysis, blood from four healthy subjects was collected, and tested in a hemolytic assay in IGP buffer (Table 4). Statistical analysis showed a significant inter-individual difference in HC_{50} values ($P < 0.005$). There were no differences between individual 1 and individual 3 ($P = 0.25$), while the HC_{50} values for individual 2 were significantly higher than for individuals 1 and 3 ($P = 0.0019$ and $P = 0.0010$, respectively), and the HC_{50} values for individual 4 were significantly lower than for individuals 1 and 3 ($P = 0.0070$ and $P = 0.0041$, respectively). It is worth noting that all four individuals tested had different blood groups according to the ABO classification system, individuals 1–4 had blood group A, B, AB, and O, respectively. One might speculate that the glycosylation pattern of erythrocyte surface antigens of e.g. the ABO system or Lewis antigens might influence the accessibility of the lipid bilayer, either by binding the peptides or by steric hindrance, thus determining the inter-individual differences in hemolytic susceptibility found in vitro.

To compare the HC_{50} values between peptides, the data from Table 4 were analyzed using 'individual' as covariant. Table 4 shows the P values of analysis of variance for comparison with histatin 5. These data demonstrate that histatin variant dhvar2 is even significantly less hemolytic than histatin 5, whereas it exhibits strongly enhanced antibacterial activity [9], demonstrating that improvement of antibacterial activity is possible without the concomitant increase in hemolytic activity.

Summarizing, in a sensitive hemolytic assay, conducted at low ionic strength isotonic conditions, subtle differences between peptides are detectable, which are undetectable in PBS. Our results emphasize that erroneous conclusions on the cell selectivity of these peptides can be drawn when the antimicrobial and hemolytic assays are not conducted in the same buffers. The relative hemolytic activity of a designed analog compared to that of a naturally occurring reference peptide is probably the best predictor for clinical cytotoxicity. As none of the designed histatin analogs were markedly more hemolytic than the naturally occurring histatin 5, these peptides might be expected to be safe candidates for antifungal treatment of superficial yeast infections.

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