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Oral Microbiology

Fungicidal effect of three new synthetic cationic peptides against *Candida albicans*

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OBJECTIVE: Peptide antibiotics are considered a new class of antifungal agents. Of these, an α-helical, cationic peptide termed Dhvar 4, a relative of salivary histatin has been shown to be an antifungal of relatively high potency. Similarly, lactoferricin B (LFB) and a derivative thereof, LFB(17–30), disrupts the fungal cell membrane and acts against *Candida albicans*. As Dhvar 4 and LFB(17–30), exhibit almost identical amino acid sequences at their C-terminal, we hypothesized that laboratory synthesis of peptides with an α-helical structure and having similar amphipathic properties could lead to products with candidacidal activity. Hence, three such peptides – JH8194, JH8195 and JH 8944, were synthesized and their antifungal properties compared with recognized antifungals LFB, LFB(17–30), human lactoferricin (LFH), Histatin-5 and Dhvar 4, against two isolates of *C. albicans*.

MATERIALS AND METHODS: The antifungal agents were synthesized and their secondary structures evaluated according to a previously described protocol of Situ and Bobek (2000) *Antimicrob Agents Chemother* 44: 1485–1493. The *C. albicans* strains were oral isolates from a human immunodeficiency virus-infected (isolate A2) and a healthy (A6) individual. A standard concentration of yeasts was exposed to a range of dilutions of the agents for a specific duration and the cell death (viability) in terms of the resultant colony forming units ml^{-1} was quantified.

RESULTS: Dhvar 4, showed the most α-helical propensity, and was the least fungicidal while LFB and LFB(17–30) showed the highest antifungal potential, and demonstrated total kill of A6, and A2 at 5 and 10 μM concentrations, respectively whilst LFH killed both isolates at a 10 μM concentration. Of the three new synthetic peptides, JH 8194 was the most potent (total kill of A6/A2 strains at 1.25/2.5 μM), followed by JH 8195 (total kill of A6/A2 strains at 5/10 μM while JH 8944 was the least potent as a 25 μM concentration was required to kill either strain of *Candida*. On further analyses of the relationship between pI value of the peptides and their anticandicidal activity, a significant positive correlation was noted. In order to rule out a cytotoxic effect of the new synthetic peptides we compared the fungicidal and hemolytic activities under similar incubation conditions using freshly isolated erythrocytes and all three peptides exhibited no detectable hemolysis upto an concentration of 100 μM in contrast to the polyene antifungal amphotericin B that elicited significant initiation of hemolysis at a concentration of 5.0 μM.

CONCLUSION: Our data suggest that laboratory synthesis of agents with an α-helical structure and having amphipathic properties similar to known, natural antifungal agents may be a promising avenue to generate products with improved antifungal activity.

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Keywords: cationic peptide; antifungal activity; *Candida albicans*

Introduction

Candida albicans is an opportunistic fungal pathogen that causes mucosal and systemic infections in humans. The AIDS epidemic, improved life-sustaining therapies, and aggressive anticancer therapy have all contributed to the rise in the number of severely immunocompromised patients in the community leading in turn to increased fungal infections because of this opportunistic yeast (Samaranayake *et al*, 2002). The concomitant escalation in the use of antifungals for prophylaxis and management of these diseases has led to the emergence of drug resistant yeasts worldwide (White *et al*, 1998). In addition, a number of currently available antifungal agents, such as polyene and azole antimycotics and DNA analogues, have undesirable toxic and other side effects (Situ and Bobek, 2000). Therefore, the search for more effective agents analogous to natural antifungal substances with lesser toxicity is of crucial importance.

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A number of natural antifungal agents are found in human mucosal secretions including saliva. These include, secretory immunoglobulin A, lactoferrin (Nikawa *et al*, 1993a, 1994, 1995), lysozyme (Tobgi *et al*, 1987; Nikawa *et al*, 1993b), and histatins (Pollock *et al*, 1984) that are demonstrably candidacidal in nature. Histatins in particular are a group of small, cationic antifungal peptides present both in human saliva (Pollock *et al*, 1984; Oppenheim *et al*, 1988) and serum (Murakami *et al*, 1994) and divided into two major sub-groups, Histatin-1 and -3 based upon the encoded gene. Of these Histatin-5, a proteolytic degradation product of Histatin-3, shows the most potent candidacidal activity (Stemberg, 1994), and is thought to play a critical role in primary protection of the oral cavity against candidal infections, especially in compromised hosts (Samaranayake, 2002). In fact, of the aforementioned proteins only the salivary histatins levels show a statistically significant decrease in AIDS patients who develop candidiasis (Tsai and Bobek, 1997). This perhaps is a reason why more than 90% of AIDS patients develop oral candidiasis over the course of the disease (Samaranayake and Holmstrup, 1989). We too have recently shown that oral *C. albicans* isolates from human immunodeficiency virus (HIV)-infected individuals are less sensitive to Histatin-5 compared with those from healthy controls (Nikawa *et al*, 2002). For these reasons, there has been intense interest in Histatin-5 and its variant synthetic peptides in order to develop compounds with more efficacious fungicidal properties. Of the synthetic variants of histatins, an α-helical peptide, Dhvar 4 has been shown to possess intense fungicidal/static activity (Helmerhorst *et al*, 1999a). Furthermore, other compounds such as lactoferricin B (LFB) and LFB(17–30), derived from bovine lactoferricin, exhibits natural antifungal activity against *C. albicans* (Bellamy *et al*, 1993; Wakabayashi *et al*, 1998) and so is another peptide derived from the N-terminal region of lactoferrin, lactoferricin (LF) (Jones *et al*, 1992; Bellamy *et al*, 1993; Lupetti *et al*, 2000). These peptide antifungals are considered a new class of promising agents that interact with the yeast cell membranes leading to the disruption of their cellular integrity and cell death (Helmerhorst *et al*, 1999b).

On comparison of the amino acid sequence of the C-terminals of Dhvar 4 and LFB(17–30) we noted a very high congruence (Table 1) and hypothesized that synthetic peptide fragments with identical amino acid sequences and amphipathic properties may yield compounds with improved fungicidal properties. Hence, we synthesized *de novo*, three different peptides – JH8194, JH8195 and JH 8944 with the latter properties, using standard biochemical techniques. Afterwards, the α-helicity and the fungicidal properties of the cationic peptides were characterized and their anticandidal activity compared with LFB, LFB(17–30), human lactoferricin (LFH), Histatin-5 and Dhvar 4, against two oral isolates of *C. albicans* derived from a healthy and a HIV-infected individual.

Table 1 Similarity of nature of comprising amino acids between LFB(17–30) and Dhavr4

Peptides	*Amino acid sequence*						
LFB(17–30)	FKC	**RR**	WQW	**R**	M	**KK**	LG
Dhvar 4	**KR**LF	**KK**	LLF	***S***	L	**RK**	Y

Letters in bold indicate basic amino acid; underline, hydrophobic amino acid; bolditalic, polar amino acid.

Materials and methods

Candida *isolates and growth conditions*

In total two isolates of *C. albicans* were used in the experiment. *Candida albicans* A2 was an oral isolates from a male, ethnic Chinese, HIV-infected individual while *C. albicans* A6 was an oral isolate from a healthy female. Both strains were isolated using an oral rinse technique as described previously (Samaranayake *et al*, 1986). The identity of both isolates was reconfirmed with standard sugar assimilation tests using the API 20C system (API Products, Biomeroux, Lyon, France) and the 'germ tube' test (Silverman *et al*, 1990). In a previous study of ours these isolates exhibited differential *in vitro* sensitivity to histatin-5, the isolate A2 being more resistant to histatin than the isolate A6 (Nikawa *et al*, 2002).

In order to obtain yeast suspensions for the sensitivity assays a loopful of the yeast was inoculated into 100 ml of yeast nitrogen base (YNB) medium (Difco, Detroit, MI, USA) containing 250 mM glucose and grown aerobically at 37°C for 24 h. After 18 h incubation, the yeasts were harvested in the mid-exponential growth phase, washed twice with phosphate-buffered saline (PBS) containing 1 mM phosphate buffer (pH 6.8) and resuspended to a final concentration of 10^7 yeasts ml^{-1} by hemocytometric counting (Nikawa *et al*, 1993a, 1996). All yeasts were in the blastospore phase at this stage.

Cationic peptides, peptide design and synthesis

Histatin-5 (DSHEKRHHGYKRKFHEKHHSHRGY; IUPAC-IUB, pI = 10.70), bovine lactroferricin (LFB; FKCRRWQWRMKKLGAPSITCVRRAF, pI = 12.34), LFB(17–30) [LFB(17–30); FKCRRWQWRMKKLG, pI = 12.23], LFH (TKCFQWQRNMRKVRGPPVSCIKR, pI = 12.07), Dhvar 4 (KRLFKKLLFSLRKY, pI = 11.69), and three new peptides, i.e. JH8194 (KRLFRRWQWRMKKY, pI = 12.51), JH8195 (KRLFRRLLFSMKKY, pI = 12.24), and JH8944 (FKCKKVVISLRRY, pI = 10.88) were all synthesized using the T-bag method adapted for 9-fluorenyl methoxycarbonyl (Fmoc)-chemistry (Helmerhorst *et al*, 1999a).

p-Benzoyloxybenzyl 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 (TANA laboratories, Houston, Texas, USA). Purification and purity analysis of peptides were performed using high performance liquid chromatography (HPLC) and reverse phase HPLC. Further confirmation of molecular weight was performed using Mass spectrometry (Matrix Assisted Laser Desorption lonization-TOF/MS).

Circular dichroism spectra analysis
Circular dichroism (CD) spectra of the peptides were acquired on a JASCO J-820 spectropolarimeter, calibrated with D10 camphor sulfonic acid. Peptides, other than Histatin-5, LFB and LFH, dissolved in 100 trifuluoroethanol (TFE), 75 TFE/distilled water (DS), 50 TFE/DS, 25 TFE/DS and 100 DS, were scanned in the range of 247–185 nm for secondary structural analysis. The peptide concentration used was 100 mg ml^{-1}. Four scans were acquired from each sample and averaged to improve signal quality. All spectra were expressed in terms of mean residue ellipticity, in units of deg cm^2 $dmol^{-1}$ (Situ and Bobek, 2000).

Secondary structure analysis
Far UV CD spectra were analyzed by convex constraint analysis (CCA) according to the method of Situ and Bobek (2000). The ellipticity values at each wavelength in the rage of 247–185 nm were used for the calculation. The input data set consisted a matrix of 30 proteins, and the spectra were deconvolved in 100 iterations. The spectral assignments of each peptide was performed as described by Perczel *et al*. (1992).

Candidacidal assay
Antifungal activity of the peptides was evaluated according to the method of Edgerton *et al* (1998) with some modifications. The assays were performed on exponential phase *C. albicans* cells in the presence or absence of increasing concentrations of the peptides (0.325–100 μM). Briefly, *C. albicans* cells were washed twice with 1 mM sodium phosphate buffer (Na_2HP0_4/NaH_2P0_4; pH 6.8), and resuspended at 1.8×10^5 cells ml^{-1}. Twenty microliters of the cell suspension was mixed with 20 ml of 1 mM phosphate buffer containing the test peptide and incubated for 90 min at 37°C with shaking. Controls were incubated with 20 ml of 1 mM phosphate buffer alone. The reaction was stopped by adding 360 ml of YNB; 40 μl of the suspension were spread onto Sabouraud dextrose agar plates and incubated for 48 h at 37°C and the resultant colony forming units (CFUs) in the test and control inocula quantified by manual counting. The assays were performed on two independent occasions with quadruplicated specimens on each occasion. Then the percent fungicidal activity of each peptide was calculated as follows.

[Colony forming units (CFU) ml^{-1} from the test suspension with the peptide CFU^{-1} ml^{-1} form the control suspension without the peptide] $\times$ 100.

Assessment of pI value of peptides
The pI (isoelectric point) value of each synthetic peptide was calculated by the use of DNASIS, a DNA and protein analysis software (Hitachi Software Engineering Co., Ltd, Tokyo, Japan.) The killing effects of each peptide at 1.25–5.0 μM were plotted against pI values, and the correlation coefficient evaluated using regression analysis.

Hemolytic assay
Human erythrocytes from a healthy donor were collected in vacuum tubes containing heparin (final concentration 20.4 U ml^{-1}) as anti-coagulant. The erythrocytes were prepared for the hemolytic assay as described by Helmerhorst *et al* (1999b). Briefly, the erythrocytes were harvested by centrifugation for 10 min at $2000 \times g$ at room temperature, and washed three times in PBS (9 mM sodium phosphate, pH 7.0 in 150 mM NaCI). From this pellet a 20% (vol/vol) of erythrocytes/PBS suspension was prepared and used directly for the hemolytic assay. A total of 100 μl of the latter suspension was added in duplicate to 100 μl of a twofold serial dilution series of each peptide in the same buffer in a 96-well V-bottomed microtiter plate. Total hemolysis (control) was achieved with 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 microtiter plate, and the absorbance measured at 450 nm. The percentage hemolysis was calculated as: [(A450 of the peptide treated sample – A450 of buffer treated sample)/(A450 of Tween-20 treated sample – A450 of buffer treated sample)] $\times$ 100. To determine the hemolysis by amphotericin B, the same assay was employed with the peptide replaced by amphotericin B.

Statistical analysis
All of the numerical data obtained were analyzed by an one-way analysis of variance (ANOVA) and subjected to Tukey's multiple range test ($P < 0.05$).

Results and discussion

Purity of synthetic peptides
Purity analysis of peptides using HPLC and Mass spectrometry revealed one major peak for each peptide, and the purity of each peptide was as follows; Histatin-5: 96.00%, LFB: 100.00%, LFB(17–30): 98.47%, LFH: 100.00%, Dhvar 4: 99.30%, JH8194: 99.55%, JH8195: 100.00%, and JH8944: 97.35%.

Conformation analysis of peptides
Secondary structure of antimicrobial peptides, particularly the α-helical conformation either in non-aqueous or a hydrophobic environment, is reported to play an important modulating role of their antimicrobial action, particularly the permeabilization of the fungal cell membrane. Thus we analyzed the α-helical propensity of each peptide in serially diluted TFE, considered an α-helix-promoting solvent. The quantitative shape analysis was performed as described by Perczel and

Table 2 Secondary structural percentage of synthetic peptides

	TFE 0%	*TFE 25%*	*TFE 50%*	*TFE 75%*	*TFE 100%*
Dhvar 4					
α-Helix	0.0	100.0	100.0	100.0	100.0
Random coil	100.0	0.0	0.0	0.0	0.0
LFB short					
α-Helix	0.0	7.4	12.1	18.3	39.0
Random coil	100.0	92.6	87.9	81.7	61.0
JH8194					
α-Helix	0.0	2.2	14.5	34.0	100.0
Random coil	100.0	97.8	85.5	66.0	0.0
JH8195					
α-Helix	0.0	17.8	100.0	100.0	100.0
Random coil	100.0	82.2	0.0	0.0	0.0
JH8944					
α-Helix	0.0	4.0	10.8	13.7	22.7
Random coil	100.0	96.0	89.2	86.3	77.3

Hollosi (1996) whereas quantitative estimation of the secondary structures was determined by CCA. The latter analysis has been previously employed to estimate the secondary structural content of oligopeptides (Perczel and Hollosi, 1996). As beta-turn is known to involve a specific pair of amino acids, and short oligopeptides usually transit between two phases, we eliminated beta-structures, such as turns or sheets, in the present study, after which the helix/random coil ratio was calculated (Table 2).

As shown in Figure 1a, Dhvar 4 showed very high α-helical propensity. In distilled water (DS), CD spectra of Dhvar 4 exhibited a random coil, and in 25% TFE/DS solution, Dhvar 4 comprised 100% α-helical conformation and 0% random coil (Figure 1a, Table 2). α-Helical conformation of Dhvar 4 matured as the TFE concentration increased from 0 to 25% (Table 2).

In contrast, CD spectra of LFB(17–30) essentially exhibited the random coil conformation, and even in 100% TFE, LFB(17–30) exhibited only 39.0% α-helical conformation and 61.0% random coil (Figure lb, Table 2). The CD spectra of JH8194 and JH 8195 showed the concentration-dependent conformational changes in TFE. JH8194 formed random coil in distilled water, and incremental increase in their α-helicity with increasing TFE concentration (α-helicity; 2.2% in 25% TFE, 14.5% in 50% TFE, and 34.0% in 75% TFE) and, total α-helical conformation in 100% TFE (Figure 1c). A similar relationship between α-helical conformation and the concentration of TFE was noted with JH8195 (Figure 1d, Table 2). In contrast, CD spectra of JH 8944 exhibited random coil conformation in 100% TFE, but this compound exhibited 22.7% α-helical conformation and 77.3% random coil in 100% TFE (Figure le, Table 2).

Fungicidal activity of peptides

Pilot studies indicated that the fungicidal effect of each peptide gradually increased up to 60 min incubation, and plateaued thereafter (data on file). Hence we examined the fungicidal effect of all the peptides after a standard 90-min period of incubation in all experiments described below.

With regard to Histatin 5 exposure the loss of viability of the two *C. albicans* isolates was dose dependent. Whilst *C. albicans* A6 was more sensitive to Histatin-5, both isolates were rendered totally non-viable at a Histatin-5 concentration of 100 μM. These observations concur with our previous studies where 50 μM Histatin-5, killed almost all (>95%) of *C. albicans* isolates from healthy individuals but only 66.1–75.3% of two *C. albicans* isolates from HIV-infected individuals. The family of salivary histatins consists of structurally related, low molecular weight histidine rich proteins that contribute to the non-immune defense system of the orol-pharyngeal milieu and, are found in saliva of healthy adults at concentrations ranging from 50–425 μg ml^{-1} (Edgerton *et al*, 1998). Histatin-5 is the most potent member of the family and renders most pathogenic *Candida* species non-viable *in vitro* at physiological concentrations (Raj *et al*, 1990).

On the contrary, Dhvar 4, a relative of histatin, which showed the most α-helical propensity, was the least effective in killing the two isolates (Figures 2 and 3). In contrast, LFB and LFB(17–30) exhibited higher potency leading to total kill of A6 and A2 isolates at 5 and 10 μM concentrations, respectively (Figures 2 and 3). The killing effect of LFH was relatively high, and 10 μM of LFH rendered both isolates totally non-viable (Figures 2 and 3). Notably the fungicidal effect of lower concentrations of LFH was less than that of LFB and LFB(17–30).

As compared with the foregoing natural peptides the JH8194, one of the three new synthetic peptides, was exquisitely candidacidal and rendered all A6 and A2 isolates non-viable at 1.25 μM at 2.5 μM concentrations. JH 8195 was of intermediate potency and killed all A2 and A6 isolates at 5 and 10 μM concentrations. However, JH 8944 was the least effective fungicidal of the three synthetic peptides, and required 25.0 μM concentrations for a total kill both isolates.

It is generally accepted that many antimicrobial peptides have an α-helical structure, and the majority are cationic and amphipathic. Our results suggest that α-helicity contributes to the killing effects of peptides, but only to a limited extent. In most microbes, including fungi, both the outer leaflet of the plasma membrane as well as the outer membrane contain anionic molecules oriented towards the exterior of the cell (Shai, 1999). As this is not the case for mammalian cell membranes, the cationic antimicrobial peptides preferentially bind to the exposed negative charges of microbial membranes, instead of the zwitterionic amphiphiles present in the exterior surface of the mammalian cells.

We further analysed the relationship between pI value of the peptides and their anticandicidal activity. A significant positive correlation between these elements was also observed (Figure 4). These results imply that higher the pI value of the peptide, the greater is its antifungal activity.

Hemolysis assay

The hemolytic activity of cationic peptides in phosphate buffer is a surrogate marker of their antimicrobial

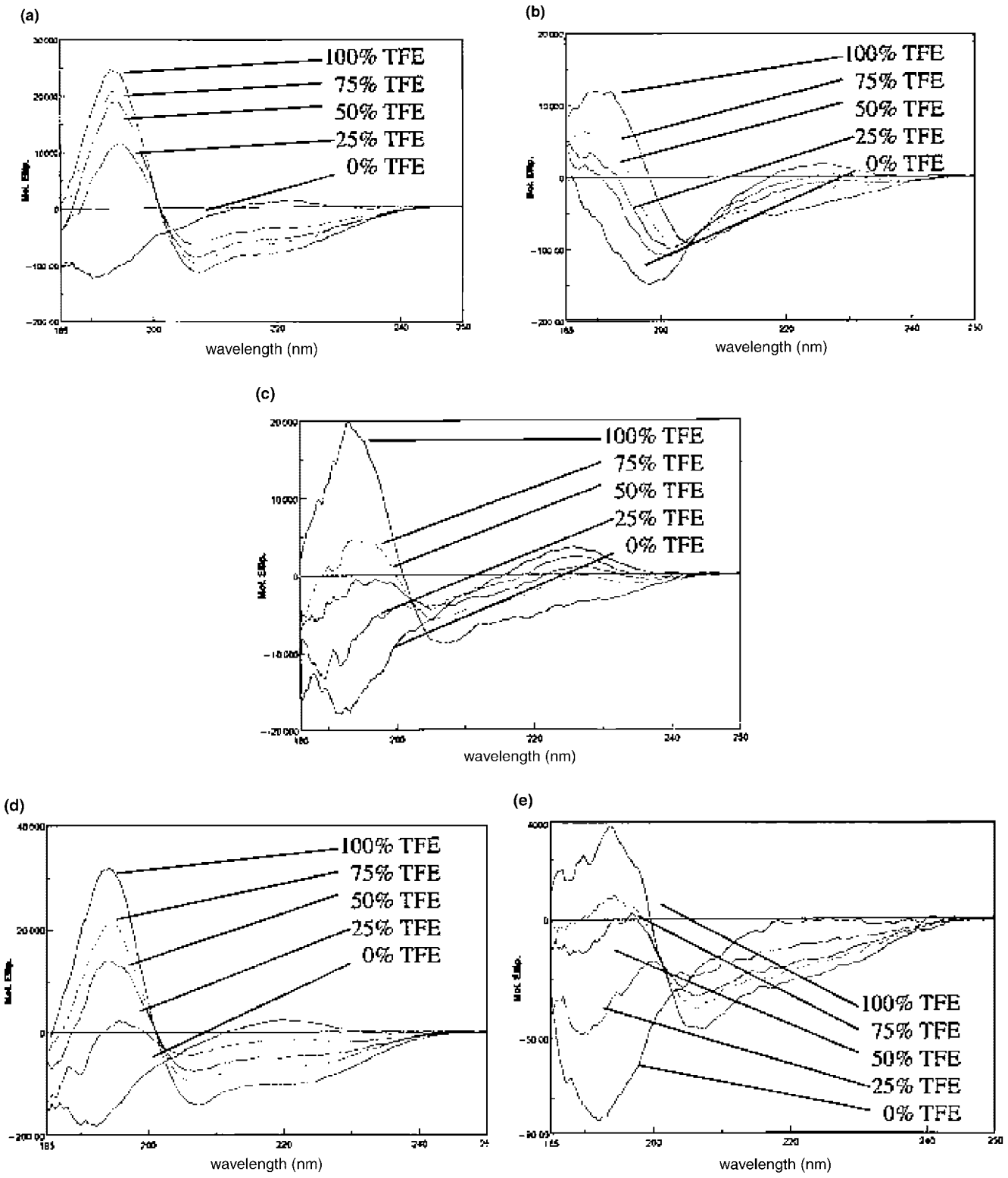

Figure 1 Conformational analysis of cationic peptides, i.e. Dhvar 4 (**a**), LFB(17–30) (**b**), JH8194 (**c**), JH8195 (**d**) and JH8944 (**e**) by circular dichroism. All spectra are expressed in terms of mean residue ellipticity. The peptide concentration was used was 100 μM

activity, in various growth media (Dathe *et al*, 1997). Yet the two assays measure different properties, as hemolysis indicates membrane perturbing properties and the latter assay, the growth inhibitory activity. We compared the fungicidal and hemolytic activities under similar incubation conditions using freshly isolated

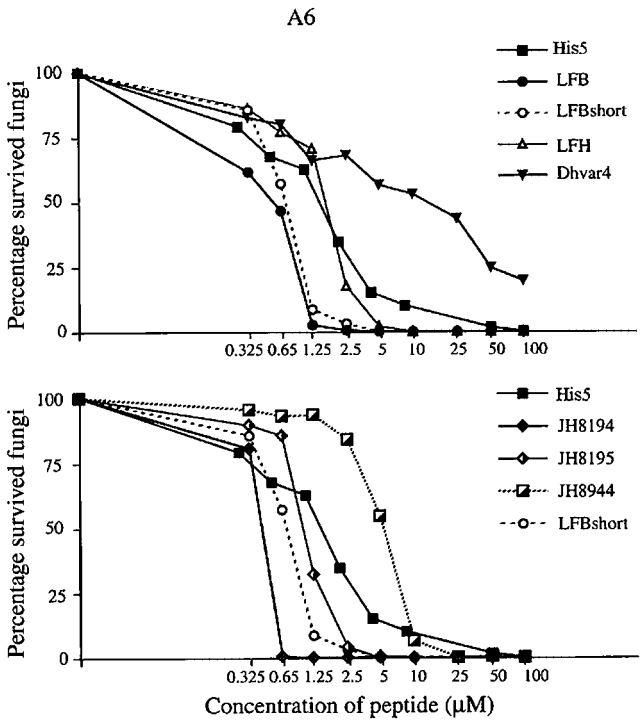

Figure 2 Killing effects of cationic peptides against *Candida albicans* A6, an isolate from human immunodeficiency virus-negative patient. The data indicate mean viability of survived fungi

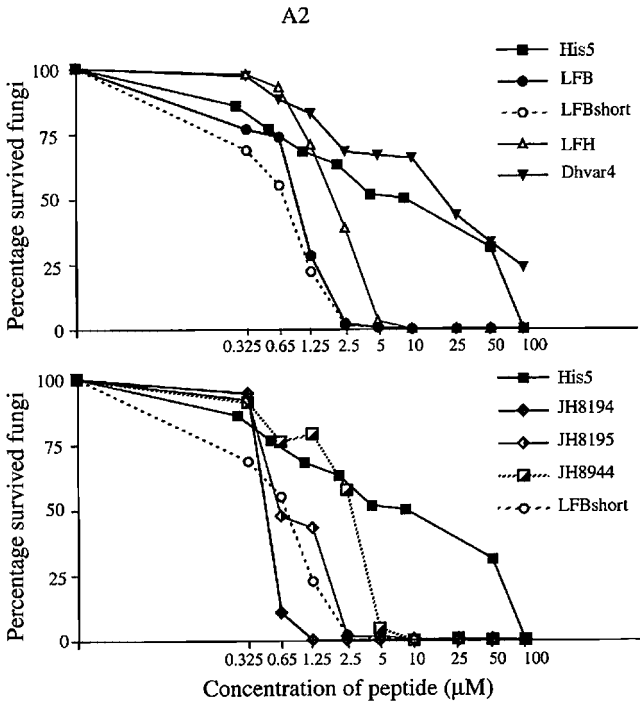

Figure 3 Cationic peptides induced loss of viability of *Candida albicans* A2, an isolate from human immunodeficiency virus-positive patient. The data indicate mean viability of survived fungi

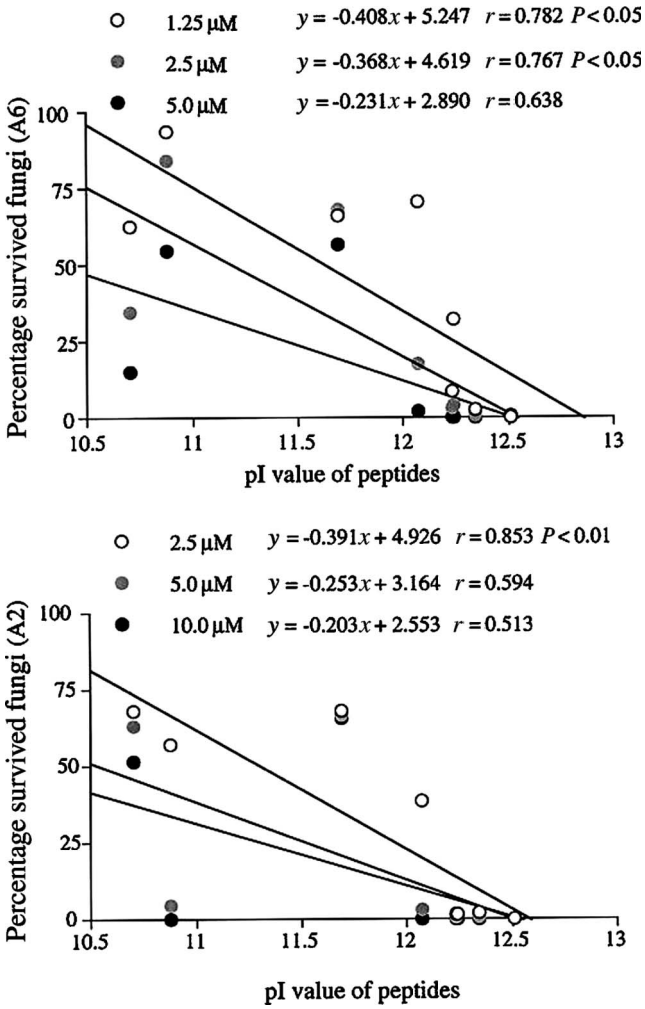

Figure 4 Relationship between pi values of cationic peptides and their fungicidal effects

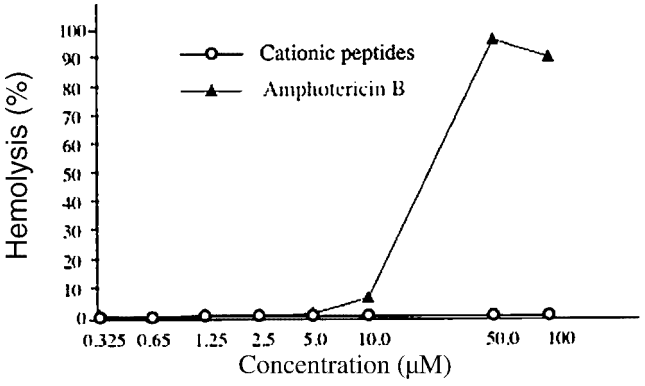

Figure 5 Hemolytic activity of cationic peptides and amphotericin B

erythrocytes as described by Helmerhorst *et al* (1999a). As shown in Figure 5, all peptides used in the present study, exhibited no detectable hemolysis upto a concentration of 100 μM in contrast to the polyene antifungal amphotericin B that elicited significant hemolysis at much lower concentrations (5.0 μM). As none of the three synthetic peptides were more hemolytic than the widely used polyene antifungal amphotericin B, these

histatin analogues could be considered as safe candidates for putative antifungal agents.

In summary, we have demonstrated that three synthetic peptides, that are histatin analogs have powerful anticandicidal activity that may be superior to the naturally occurring antifungals such as histatin 5, lactoferricin B (LFB) and its derivative LFB(17–30), and the synthetic peptides Dhvar 4 The pI values of these synthetic peptides indicate that they may be further developed as efficacious agents for treating candidal infections.

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