Human salivary MUC7 mucin peptides: effect of size, charge and cysteine residues on antifungal activity

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We have previously shown that MUC7 (human salivary low-molecular-mass mucin) 20-mer: LAHQKPFIRKSYKCLHKRCR (residues 32–51 of the parent MUC7, with a net positive charge of 7) possesses a broad-spectrum antimicrobial activity [Bobek and Situ (2003) *Antimicrob. Agents Chemother.* **47**, 645–652]. The aims of the present study were to determine the minimum peptide chain length and its location within the 20-mer region that retains potent antifungal activity against *Candida albicans* and *Cryptococcus neoformans* and to examine the effect of net charge of the peptide as well as the role of cysteine residues on the fungicidal activity. First, several C-terminal truncated MUC7 20-mer fragments (16-mer, 12-mer, 11-mer, 10-mer and 8-mer) and one N-terminal fragment (8-mer-N) were synthesized and tested. The results showed that MUC7 12-mer, located at the C-terminal region of MUC7 20-mer, having a net charge of +6 and exhibiting an amphipathic helical conformation, not only retained

but exceeded the antifungal activity of that of 20-mer. Secondly, several variants of the 12-mer peptide containing a lower or no net positive charge, or no cysteine residues were synthesized and tested. A clear correlation between the net positive charge of the 12-mer, its potency and initial interaction of peptide with fungal cells was found by killing assays, fluorescence microscopy and fungal cell-membrane potential measurements. Furthermore, cysteine residues, which play a critical role in bacterial binding, were found to be not important for the fungicidal activity of these peptides. These results identified MUC7 12-mer as a potential candidate for development into a novel antifungal therapeutic agent.

Key words: amphipathicity, *Candida albicans*, *Cryptococcus neoformans*, MUC7 12-mer, secondary structure.

INTRODUCTION

The occurrence of fungal infections has increased significantly in the past decade and is expected to continue to increase in the foreseeable future. Hosts for which there is evidence of increased risk of fungal infections are the classic immunocompromised patients (including HIV/AIDS patients, oncology patients with neutropenia, bone marrow and organ-transplant recipients) and non-immunocompromised critically ill patients [1]. The toxicity of some of the presently available antifungal agents and the emergence of drug-resistant fungal strains are the major disadvantages of the current antifungal therapies. Thus development of new antimicrobial agents with different mechanisms of action, low toxicity and low tendency to elicit resistance is urgently needed.

In the past few years, CAMPs (cationic antimicrobial peptides) have received increasing attention as a promising source of novel antimicrobial compounds [2–5]. This is because unlike many currently used antimicrobials, CAMPs show little or no toxicity towards mammalian cells and low tendency to elicit resistance [6,7]. Several hundred different natural (gene-encoded) CAMPs have been identified. They are produced by living organisms of all types, including humans and plants. They are mobilized shortly after infection as part of the innate immunity and act rapidly to neutralize a broad range of microbes, including Gram-negative and Gram-positive bacteria, antibiotic-resistant bacteria, fungi, viruses and parasites [8,9]. In addition, thousands of synthetic CAMP variants have been synthesized [10]. The functional and structural properties and therapeutic potential of antimicrobial peptides have been recently reviewed [5,11–15]. A low mass (molecular mass between 1 and 5 kDa), amphipathic structure

(separate hydrophobic and hydrophilic domains) in non-polar solvent and net positive charge are the common features of most naturally occurring antimicrobial peptides. Most of these peptides exhibit a random structure in water and a well-defined structure (α -helical, β -sheet, extended structures and loops) in a simulated membrane-like environment [6,14,16]. Based on their secondary structures, antimicrobial peptides are commonly classified into four groups. Group I comprises linear peptides with α -helical structure (examples include amphibian buforin, insect and pig cecropins, frog magainins and human cathelicidin LL-37). Group II comprises linear peptides with an extended structure (examples include bovine and pig cathelicidins, proline-rich peptides produced by all classes of animals and human histatins). Group III comprises peptides containing loop structure and one disulphide (S–S) bond (examples are bovine bactenecin, bovine and human lactoferricin). Group IV consists of β -strand peptides connected by intramolecular S–S bridges (examples are pig protegrins, human α -defensins, human and other mammals' β -defensins). With respect to structure and function, magainins, dermaseptins, cecropins, mammalian defensins, lactoferricin and histatins have been best characterized [14].

We have been encouraged and motivated to pursue further the antimicrobial activity of one such peptide based on our previous findings. We have shown previously that MUC7 (human salivary low-molecular-mass mucin) 20-mer (residues 32–51 of the parent MUC7, with a net positive charge of 7) possesses a broad-spectrum antimicrobial activity *in vitro* [17]. It is effective against a variety of fungi (including *Candida albicans* and *Cryptococcus neoformans*), Gram-positive bacteria (including the cariogenic *Streptococcus mutans*) and Gram-negative bacteria (including the

Abbreviations used: CAMP, cationic antimicrobial peptide; DiSC₃(5), 3,3'-dipropyl-2,2'-thiadicyanocyanine iodide; Hsn5 12-mer, a peptide derived from histatin 5 parent molecule; MUC7, low-molecular-mass human salivary mucin with a net positive charge of 7; rNMUC7, recombinant protein consisting of the N-terminal 144 residues of MUC7; RM, reductive methylation; SAB, Sabouraud dextrose agar; TFE, trifluoroethanol.

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Table 1 Amino acid sequences and ED₅₀ of unaltered MUC7 20-mer and truncated peptides

Peptide	Amino acid sequence*	Net charge†	ED ₅₀ at 95 % confidence limit (μM)	
			<i>C. albicans</i>	<i>C. neoformans</i>
MUC7 20-mer	L ³² AHQKPFIRKSYKCLHKRCR ⁵¹	7 +	5.8 (4.2–8.7)	6.7 (6.1–7.4)‡
16-mer	KPFIRKSYKCLHKRCR	7 +	6.9 (4.8–9.5)	5.3 (4.1–6.7)
12-mer	RKSYKCLHKRCR	6 +	2.1 (1.3–3.2)	2.1 (1.8–2.5)
11-mer	KSYKCLHKRCR	5 +	3.3 (1.9–5.6)	4.5 (2.6–14.2)
10-mer	SYKCLHKRCR	4 +	20.7 (17.7–24.6)	5.6 (2.3–19.4)
8-mer	KCLHKRCR	4 +	23.2 (13.4–58.6)	5.8 (2.7–15.8)
8-mer-N	LAHQKPF	1 +	No activity	No activity
Magainin II	G ¹ IGKFLHSAKKFGKAFVGEIMNS ²³	3 +	5.7 (4.0–8.4)	4.4 (3.5–5.6)
Insulin chain A	G ¹ IVEQCCASVCSLYQLENYCN ²¹	2 –	No activity	No activity

* Numbers in MUC7 20-mer sequence indicate residue numbers of the native MUC7.

† Net charge of peptides at pH 7.0.

‡ Data on MUC7 20-mer are from our previously published study [17].

Table 2 Amino acid sequences and ED₅₀ of MUC7 12-mer and its variants

Peptide	Amino acid sequence*	Net charge†	ED ₅₀ at 95 % confidence limit (μM)	
			<i>C. albicans</i>	<i>C. neoformans</i>
MUC7 12-mer	R ⁴⁰ KSYKCLHKRCR ⁵¹	6 +	2.1 (1.3–3.2)	2.1 (1.8–2.5)
12-mer-2	AASYKCLHKRCR	4 +	4.9 (4.0–5.9)	4.5 (3.1–6.2)
12-mer-3	AASYACLHAACA	0	No activity	No activity
12-mer-4	RKSYKALHKRAR	6 +	2.0 (0.8–3.7)	2.5 (1.6–3.9)
12-mer-5	RKSYKCLHKACA	4 +	5.1 (4.4–5.9)	4.9 (4.3–5.5)
Hsn5 12-mer	A ⁴ KRHGGYKRFH ¹⁵	5 +	3.7 (2.4–5.0)	2.6 (0.5–4.9)

* Underlined residues indicate substitutions in the sequence. Numbers in the MUC7 12-mer and Hsn5 12-mer sequences indicate residue numbers of the native MUC7 and Hsn 5 respectively.

† Net charge of peptides at pH 7.0.

periodontopathic *Porphyromonas gingivalis*). The antifungal activity was inhibited by bivalent cations, but not inhibited by a low temperature. Secondary-structure prediction showed that MUC7 20-mer adopts an amphiphilic helical conformation with distinguished hydrophilic and hydrophobic faces; it thus falls into group I of antimicrobial peptides (described above). Fluorescence microscopy showed the ability of MUC7 20-mer to cross the fungal cell membrane and to accumulate inside the cells where it acts, quite probably, on a specific part of the subcellular machinery. The internalization of MUC7 20-mer was inhibited by bivalent cations [17]. Thus we hypothesized that amphipathicity and high net positive charge of MUC7 20-mer are the characteristics associated with its antifungal potency. Furthermore, the mechanism of antifungal action of a 20-mer involves initial electrostatic interactions between the positively charged residues of the peptide and the negatively charged lipid head groups of the fungal cell membrane (consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and ergosterol). The bivalent cations would diminish the electronegative potential of the membrane, thus reducing and/or eliminating the cytotoxicity of the peptide.

The present study was undertaken to: (1) determine the minimum peptide chain length and its location within the MUC7 20-mer region that retains antifungal activity comparable with that of 20-mer; (2) examine the effect of peptide charge on the

fungicidal activity of MUC7-derived peptides; (3) examine the initial interaction of the peptides with fungal cells by fluorescent microscopy and transmembrane potential measurements; and (4) examine the effect of cysteine residues on the fungicidal activity of MUC7-derived peptides. The preliminary results on the antifungal activity of MUC7 20-mer truncated peptides were published in an abstract form [18].

EXPERIMENTAL

Materials

SAB (Sabouraud dextrose agar) was from Difco Laboratories (Detroit, MI, U.S.A.). Insulin chain A (21 amino acid residue peptide) and Magainin II (23 amino acid residue peptide) were purchased from Sigma (St. Louis, MO, U.S.A.). MUC7 20-mer (amino acid residues 32–51 of the parent MUC7) and its truncated and altered peptides (Tables 1 and 2), Hsn5 12-mer (amino acid residues 4–15 of a peptide derived from histatin 5 parent molecule; see Table 2) and FITC-labelled MUC7 20-mer and FITC-labelled MUC7 12-mer-3 (variants of MUC7 12-mer) peptides were all custom-synthesized by Bio-Synthesis (Lewisville, TX, U.S.A.). HPLC and MS assays were performed by the company to analyse the purity of the peptides. In general, the purity of the peptides was between 80 and 99%. The purity was taken into consideration in

the preparation of the stock solution of each peptide for antifungal assays. DiSC₃(5) (3,3'-dipropyl-2,2'-thiadicarbocyanine iodide) was purchased from Molecular Probes (Eugene, OR, U.S.A.).

Strains and culture conditions

A clinical isolate of *C. albicans* was a gift from Dr Theodore C. White (University of Washington and Seattle Biomedical Research Institute, Seattle, WA, U.S.A.). *C. neoformans* (CN2) strain was obtained from an AIDS patient with cryptococcal meningitis and was generously provided by Dr John H. Rex (University of Texas Medical School, Houston, TX, U.S.A.). Fungi were streaked and grown on SAB plates at 37 °C until large colonies were formed. One colony was then picked and resuspended in 10 mM sodium phosphate buffer (pH 7.4) and the concentration was adjusted to 1×10^5 cells/ml for the antifungal assay described below.

Antifungal activity assays

Two-fold serial dilutions of each peptide (ranging from 1.56 to 50 μ M for *C. albicans* and 1.56 to 25 μ M for *C. neoformans*) in 20 μ l of 10 mM sodium phosphate buffer (pH 7.4) were incubated with an equal volume of *C. albicans* or *C. neoformans* (10^5 cells/ml, also in 10 mM sodium phosphate buffer, pH 7.4) for 1.5 h at 37 °C. At the end of the incubation, the samples were diluted 20-fold with the same buffer and 50 μ l of aliquots (approx. 150 cells) of each sample were plated on SAB plates. Plates were incubated for 1 or 2 days for *C. albicans* and *C. neoformans* respectively. Colonies were then counted and the loss of cell viability was plotted as a function of protein concentration.

Statistical analysis

ED₅₀ values (molar concentrations of peptides required to kill half of the maximal number of cells) and 95 % confidence limits of ED₅₀ values were determined by the procedure PROBIT (SPSS software package 6.1.2 for Macintosh).

Secondary-structure prediction and CD spectral analysis

For secondary-structure prediction, we utilized the PSI Pred graphical viewer by Brunel Bioinformatics Group of Brunel University (Uxbridge, Middx., U.K.). The helical wheel projections of the predicted helical region of each peptide were made using GCG Sequence Analysis software.

CD spectra were acquired on a JASCO J715 spectropolarimeter, calibrated with d10 camphor sulphonic acid. Samples (15 μ g/ml) were dissolved in 10 mM sodium phosphate buffer (pH 7.4) or 100 % TFE (trifluoroethanol) and spectra were scanned in the range of 260–190 nm at 20 °C using a cell of 1.0 cm path length. All CD spectra are expressed in ellipticity in millidegrees.

Fluorescence light microscopy

The internalization of MUC7 20-mer and 12-mer-3 into fungi was visualized using FITC-labelled peptides. Cells (10^7) were treated with 50 μ M FITC–MUC7 20-mer or 12-mer-3 for 45 min in 100 μ l of sodium phosphate buffer (10 mM, pH 7.4) at 37 °C. The cells were then extensively washed with the same phosphate buffer, concentrated by centrifugation and resuspended in the same buffer. The cell suspension was then quickly mounted on slides with sealed coverslips. Fluorescence light micrographs

were obtained using a Nikon Optiphot microscope, with a fluorescent light source.

Analysis of changes in fungal transmembrane potential

Peptide-induced permeabilization of *C. albicans* and *C. neoformans* cell membranes was monitored by measuring changes in transmembrane potential. Measurements were taken using the transmembrane potential-sensitive fluorescent dye DiSC₃(5). Experiments were performed using a PerkinElmer LS 50 B spectrofluorimeter kindly provided by Dr John M. Canty (School of Medicine, University at Buffalo, NY, U.S.A.) in a 700 μ l fluorescence cuvette connected to a 37 °C water bath. Fungi were grown overnight on SAB plates and resuspended in 10 mM sodium phosphate buffer (pH 7.4) at a concentration of 1×10^5 cells/ml (same conditions as for fungicidal assays) and placed into a cuvette. Dye (diluted in ethanol) was then added to the cells to achieve a final concentration of 714 nM. Ethanol in such a low dilution does not have any significant effect on the killing of the cells. Samples were monitored at excitation and emission wavelengths of 633 and 666 nm respectively. After internalization of the dye into cells (at approx. 10 min; shown by the lowest point in the fluorescence), the peptides were added at concentrations of 18 μ M. Changes in fluorescence density were monitored further up to 1500 s (25 min). Results were graphically displayed as a function of fluorescence intensity in arbitrary units against time.

RESULTS

Effect of peptide size on fungicidal activity

To determine the minimum peptide chain length and its location within the MUC7 20-mer required for antifungal activity comparable with that of 20-mer, we first tested a series of truncated peptides. We decided to make the truncation at the N-terminus of the 20-mer (with the net positive charge of 7) since the N-terminus lacks positively charged residues. We began the analysis with five N-truncated peptides (see Table 1): 16-mer, 12-mer, 11-mer, 10-mer and 8-mer, with net positive charges of 7, 6, 5, 4 and 4 respectively. The fungicidal activities of these peptides (dose–response curves) against *C. albicans* and *C. neoformans* are shown in Figure 1. Since these peptides are more effective against *C. neoformans* than *C. albicans*, the peptide concentration axes for *C. neoformans* is scaled only up to 25 μ M (rather than 50 μ M) to see better the dose–response differences at lower peptide concentrations. The ED₅₀ values for the MUC7 peptides in comparison with a well-characterized CAMP Magainin II (23 amino acid residues) [19] and a negative control peptide insulin chain A (21 amino acid residues) are shown in Table 1.

Our results indicate that 16-mer (with the net positive charge of 7, same as the 20-mer) has an activity comparable with that of 20-mer against both the *C. albicans* (ED₅₀ of 6.9 μ M for 16-mer versus 5.8 μ M for 20-mer) and *C. neoformans* (ED₅₀ of 5.3 μ M for 16-mer versus 6.7 μ M for 20-mer). The activities of both MUC7 20-mer and 16-mer are also comparable with that of Magainin II (95 % confidence limits of all ED₅₀ values overlap). On the other hand, insulin chain A showed no activity. Interestingly, compared with 20-mer and 16-mer, 12-mer (with the net positive charge of 6) possesses increased activity against both fungi (showing ED₅₀ of 2.1 μ M for both). The activity of 11-mer (with the net positive charge of 5) slightly decreased compared with that of 12-mer. The activities of 10-mer and 8-mer (net positive charge of 4 for both) against *C. albicans* markedly decreased (ED₅₀ of

Figure 1 Fungicidal activity of MUC7 16-mer and other truncated peptides

Fungal cells (2×10^5 in $20 \mu\text{l}$ of 10 mM sodium phosphate buffer) were incubated with $20 \mu\text{l}$ of peptide [concentrations ranging from 1.56 to $25 \mu\text{M}$ (or $50 \mu\text{M}$) in 10 mM sodium phosphate buffer] for 1.5 h at 37°C . Cell viability was determined by plating on SAB plates. Results represent the mean for duplicate or triplicate experiments.

20.7 and $23.2 \mu\text{M}$ respectively). On the other hand, the activity of 10-mer and 8-mer against *C. neoformans* remained comparable with that of 11-mer (95% confidence limit of the ED_{50} values for all three peptides overlap). This may be because the cell surface of *C. neoformans* is highly negatively charged (more so compared with *C. albicans*) owing to its polysaccharide capsule and melanin [20], which may favour an association with the MUC7 cationic peptides. Because even the 8-mer possessed considerable activity against both fungi, but in particular against *C. neoformans*, we wondered if a peptide of the same length from the N-terminus of 20-mer would also show antifungal activity. Thus the N-terminal 8-mer (with a net positive charge of 1) was custom-synthesized and tested. As shown in Figure 1 and Table 1, this 8-mer-N showed no antifungal activity. From the above experiments we concluded that the 12-mer, the C-terminal fragment of the 20-mer, which not only retained but also exceeded the antifungal activity of the 20-mer, is the optimal peptide fragment that possesses the strongest antifungal activity.

Figure 2 Fungicidal activity of unaltered MUC7 12-mer peptide and its variants

Details are the same as in the legend to Figure 1.

Effect of charge on MUC7 12-mer fungicidal activity

Several modified peptides of the 12-mer were synthesized and tested to determine the importance of its structural features, in particular the net charge. The fungicidal activities (dose-response curves) of several MUC7 12-mer variants in comparison with the unaltered MUC7 12-mer are shown in Figure 2. The ED_{50} values of these peptides in comparison with the Hsn5 12-mer are shown in Table 2. Hsn5 12-mer (P113) is a 12 amino acid-residue fragment of Hsn5 (residues 4–15) that was previously shown to be the smallest fragment retaining the anticandidal activity comparable with that of full-length Hsn5 [21]. MUC7 12-mer has a net positive charge of 6 (versus 7 in the 20-mer). Its ED_{50} values against both fungi are $2.1 \mu\text{M}$. In comparison with MUC7 12-mer, Hsn5 12-mer (net positive charge of 5) showed slightly lower activity against *C. albicans* but a comparable activity against *C. neoformans* (Table 2).

In the first variant 12-mer-2, two positively charged amino acid residues (arginine and lysine) at the N-terminus were substituted with alanine, decreasing the net positive charge of the peptide from 6 to 4. In comparison with the unaltered 12-mer, the ED_{50}

Figure 3 CD spectra and helical wheel projections of MUC7 12-mer peptides

CD spectra (A) were obtained in 100% TFE as described in the Experimental section. Helical wheel projections (B) were made using GCG Sequence Analysis software. They were based on the 20-mer (LAHQKPFIRKSYKCLHKRCR) α -helical prediction, where 12 amino acid residues (FIRKSYKCLHKRCR) were predicted to be α -helical [17]. The 12-mer (RKSYKCLHKRCR), depicted on the left side, is the C-terminal region of the 20-mer, where 10 amino acid residues are in α -helical conformation. In the 12-mer-2, depicted on the right side, the first two amino acid residues, RK, were substituted with AA (indicated by arrows). Basic amino acid residues are displayed in black letters in open circles.

values of this variant against both *C. albicans* and *C. neoformans* approximately doubled (Table 2) and the values are comparable with that of 20-mer (the ED₅₀ values overlap). To determine if the position of the positive charges in the peptide play a role in its activity, a peptide with an equivalent deletion of two positive charges at the C-terminus (12-mer-5) was synthesized and tested. This variant showed an activity comparable with the one at the N-terminus (12-mer-2; 95% confidence limit values of the two peptides overlap), indicating that the position of the positive charges does not play a role in the activity. Activities of both these variants against *C. albicans* are higher than that of 8-mer, which has the same net positive charge (+4), indicating that both the charge and size are important for the optimal activity. Replacement of all six positively charged amino acid residues of MUC7 12-mer with alanine (12-mer-3), resulted in zero net charge and in a complete loss of antifungal activity (Table 2).

CD spectroscopy and secondary-structure predictions of MUC7 peptides

The CD spectra of several MUC7 12-mer peptides obtained in TFE (a membrane mimetic environment), in comparison with those of MUC7 20-mer and Hsn5 12-mer are shown in Figure 3(A). Similar approaches (CD spectroscopy and helical wheel projection) have been used in related studies using Hsn5 peptide fragments [21,22].

We have determined previously [17] and confirmed in the present study that MUC7 20-mer adopts an α -helical conformation

in TFE. Similarly, all MUC7 12-mer peptides show a tendency to adopt the helical conformation. This is exhibited by a CD spectrum with a positive band below 200 nm and negative bands at 206–208 nm and approx. 220 nm (on the other hand, in aqueous buffer, MUC7 20-mer and 12-mer exhibited spectra characteristic of polypeptide random coils; results not shown). To compare the tendency of MUC7 20-mer and of the other peptides investigated in the present study to adopt helical structures, we used *R* values ($R = \theta_n - \pi^* / \theta_{\pi} - \pi^*$), the ratio of the two negative bands (220/206–208). This is because using absolute ellipticity values to estimate the helical content of peptides of low molecular mass has been reported to underestimate the helical content [23,24]. An *R* value of approx. 1.00 has been assigned to α -helical peptides. The *R* value for MUC7 20-mer is 0.86, indicating a strong tendency to adopt α -helical conformation; *R* values for MUC7 12-mer and Hsn5 12-mer are 0.56 and 0.55 respectively, also indicating populations of α -helical conformation in each of these peptides.

Previously, we also showed by a secondary-structure prediction and helical wheel projection that MUC7 20-mer adopts an amphiphilic helical conformation with distinguished hydrophobic and hydrophilic faces, thought to be important for antimicrobial activity [17]. A secondary-structure prediction (based on the 20-mer) indicated that the ten N-terminal residues of the 12-mer are in a helical conformation. A helical wheel projection of MUC7 12-mer depicted in Figure 3(B) (left side) shows that this peptide, similar to MUC7 20-mer [17], also adopts an amphiphilic helical conformation with a distinguished hydrophobic and a hydrophilic face. This prediction was confirmed by CD spectroscopy in TFE, as shown in Figure 3(A).

MUC7 12-mer-2 has a similar tendency to adopt α -helical conformation to that of unaltered 12-mer with an *R* value of 0.6. However, this substitution adds more hydrophobic residues and somewhat disrupts the hydrophilic phase of the helix (Figure 3B, right side). The CD spectrum of 12-mer-3 (all positively charged residues substituted by alanine) indicated that this peptide exhibits even stronger tendency to adopt α -helical conformation than the unaltered 12-mer (*R* value of 0.79). Even though these amino acid substitutions completely disrupted the amphipathicity, they increased the hydrophobicity and thus the tendency to adopt an helical structure. These results indicate that the amphipathic nature of the peptide and higher net positive charge are important for the antifungal activity.

Effect of cysteine residues on fungicidal activity of MUC 12-mer

It was previously reported that rNMUC7 (recombinant protein consisting of the N-terminal 144 residues of MUC7) bound to *S. mutans* [25]. Reductive methylation (RM) of rNMUC7 (lysine residues were converted into mono- or dimethyl lysine, as confirmed by the amino acid analysis) had little or no effect on *S. mutans* binding. When RM sample was reduced further and alkylated (RM/RA; alkylation performed by iodoacetic acid; cysteine residues were quantitatively converted into carboxymethyl cysteine residues, as confirmed by amino acid analysis), no binding was detected. When the rNMUC7 was just reduced and alkylated (RA), again, no binding was detected. The authors concluded that alkylation of the two cysteine residues (Cys⁴⁵ and Cys⁵⁰) resulted in the complete loss of bacterial binding [25]. To examine if the two cysteine residues play a role in the fungicidal activity of MUC7 12-mer, 12-mer-4 with the two cysteine residues substituted with alanine was designed and custom-synthesized. Interestingly, this substitution did not alter the 12-mer antifungal activity (Figure 2 and Table 2), indicating that the cysteine residues are not important for fungicidal activity against both

Figure 4 Fluorescence light microscopy of internalization of MUC7 peptides by *C. albicans* and *C. neoformans*

Cells (10^7) were treated with 50 μ M of FITC-MUC7 20-mer (**A, D**) or 50 μ M of FITC-MUC7 12-mer-3 (**B, C, E, F**) for 45–90 min in 100 μ l of sodium phosphate buffer (10 mM, pH 7.4) at 37 °C. After extensive washing, cells were examined by light microscopy using a Nikon Optiphot microscope, with a fluorescent light source. Cells treated with FITC-12-mer-3 were also examined by transmission microscopy (**C, F**, bright field).

C. albicans and *C. neoformans*. By CD spectroscopy, this peptide shows a tendency to adopt an α -helical structure similar to that of unaltered peptide, with the R value of 0.55.

Visualization of binding of MUC7 peptides and internalization

We hypothesized that the initial mechanism of antifungal action of the MUC7 20-mer involves initial electrostatic interactions between the positively charged residues of the peptide and the negatively charged head groups of the fungal cell membrane. Therefore with no positively charged residues, the interaction of 12-mer-3 with fungal cells would be completely abolished. Our fluorescence light microscopy study confirmed our hypothesis (Figure 4). After incubating the fungal cells (*C. albicans* and *C. neoformans*) with FITC-12-mer-3, this peptide was not visible either on the cell membrane or inside of both the *Candida* (Figure 4B) and *Cryptococcus* cells (Figure 4E), whereas the FITC-20-mer was visible (Figures 4A and 4D). These results showed that MUC7 12-mer-3, with zero net positive charge, is not able to interact with the fungal cell membrane and subsequently be taken up by the cells. The transmission microscopy (bright field) of cells treated with FITC-12-mer-3 assured the presence of fungal cells (Figures 4C and 4F).

Effects of MUC7 peptides on fungal cell-membrane depolarization

Relative changes in transmembrane potentials of *C. albicans* and *C. neoformans* were monitored as MUC7 peptides, Hsn5-12-mer and insulin chain A were added to the cells preloaded with a membrane potential-sensitive fluorescent dye, DiSC₃(5), as described in the Experimental section. Similar approach was used by other investigators using Hsn5 and Hsn5 variants [22,26]. This dye distributes between cells and suspension medium depending on the membrane potential gradient. It is fluorescent in solution but when taken up by the cells, it aggregates and self-quenches [27]. On permeabilization of the membrane by peptides, the transmembrane potential changes due to the flux

Figure 5 Effects of MUC7 peptides on transmembrane potential of *C. albicans*

Alterations in transmembrane potential were measured by release of DiSC₃(5) dye from fungal cells on the addition of peptides to a final concentration of 18 μ M. Traces were recorded by an LS 50 B PerkinElmer spectrofluorimeter at 37 °C. To a suspension of cells (690 μ l of 10^8 cells/ml in 10 mM sodium phosphate buffer, pH 7.4) in a 700 μ l cuvette, 10 μ l of dye was added to final concentration of 714 nM (shown by an initial peak of fluorescence). Internalization of the dye by the cells is shown by a decrease in fluorescence. When a constant fluorescence was achieved (at approx. 600 s, shown by the lowest fluorescence), peptides were added. Release of the dye, shown by an increase in fluorescence, was monitored up to 1500 s. Relative fluorescence intensity is depicted in arbitrary units.

of ions, triggering release of DiSC₃(5) dye from the cells into the medium. The released dye regains its fluorescent properties outside the cells and is detected by an increase in fluorescence reading monitored by spectrofluorimeter.

To observe the dissipation of membrane potential immediately after the addition of peptides, relatively high doses of peptides (18 μ M or 3–8-fold of their ED₅₀ values) were used for this experiment. The results, depicted in Figure 5, showed that MUC7 20-mer and 12-mer possess membrane-perturbation activity. This

is evidenced by the release of DiSC₃(5) probe on addition of these peptides to the cells, measured by an increase in the fluorescence signal (only the results obtained with *C. albicans* are shown; however, similar patterns were observed with *C. neoformans*). Experiments involving Hsn5-12-mer peptide also showed a notable increase in the fluorescence signal, indicating release of the dye into the medium. On the other hand, MUC7 12-mer-3 (with zero net positive charge), which has no antifungal activity, showed little or no release to DiSC₃(5) dye (indicated by a solid line in Figure 5) and thus had no effect on the cell-membrane potential. This result correlates well with the result obtained for the FITC-labelled MUC7 12-mer-3, showing no internalization of this peptide in the fungal cells (Figure 4). The control peptide, insulin chain A, which had no antifungal activity, also showed no effect on the fungal cell-membrane potential (Figure 5).

DISCUSSION

Salivary mucins are important components of the non-immune innate host-defence system. They contribute to the formation of a protective film on both soft and hard tissues of the oral cavity and play a role in the modulation of microbial flora [28–30]. They do not directly kill micro-organisms, but have been found to interact with respiratory (e.g. *P. aeruginosa*), cariogenic (e.g. *S. mutans*) and periodontal pathogens (e.g. *P. gingivalis*), the opportunistic yeast *C. albicans* and even with HSV-1 and HIV-1 viruses [31]. Recombinant full-length MUC7 apo-protein (357 amino acid residues) did not show any appreciable candidacidal activity (ED₅₀ of 280 μ M; see [32]). However, the peptides derived from its N-terminal region have a significant and a broad-spectrum fungicidal and bactericidal activity *in vitro* [17,18,25,32,33]. There is also a possibility that MUC7 peptides from the N-terminal end can be generated *in vivo* by degradation of the parent MUC7 molecule by proteolytic enzymes present in human saliva, since the N-terminal region is free of glycosylation, and thus susceptible to proteolysis. Free in saliva, these peptides could serve as a part of the innate defence system against pathogens. In fact, a recent study [34] revealed a 20 kDa MUC7 fragment present in total saliva that contained a portion of an N-terminal region of MUC7, suggesting that cleavage of MUC7 *in vivo* might yield fragments with microbicidal properties. A previous study [32] had analysed whether whole saliva or *C. albicans* cells are capable of degrading the MUC7 parent molecule to smaller fragments. A radiolabelled MUC7 was used and its degradation was monitored by SDS/PAGE. The exposure of MUC7 to saliva resulted in its considerable degradation (after 12 h, almost a complete loss of the intact MUC7 band was observed); a lower rate of degradation of MUC7 was noted in the presence of *C. albicans*, indicating that saliva contains a higher level of proteolytic enzymes than *C. albicans*, and hence is also capable of degrading MUC7. At present in our laboratory, we are actively pursuing the identification of the generated fragments. In the present study, however, we stress the point of considering the MUC7 peptides as candidates for development into novel antimicrobial agents to be used for therapeutic purposes *in vivo*, including their use as salivary substitutes. In that regard, MUC7 12-mer is an optimal candidate.

For a better definition of the factors affecting the specific antifungal activity of MUC7 peptides, in the present study, we investigated the relationship between factors, such as peptide chain length, net positive charge, amphipathicity and its fungi-killing ability. We first determined the minimum peptide chain length and its location within the MUC7 20-mer required to retain the antifungal activity comparable with that of the 20-mer. A series of truncated peptides derived from 20-mer were designed

and prepared. The antifungal activity of MUC7 20-mer appears to be in the C-terminal half, and in fact, discarding the N-terminal region increased the activity. Since the 8-mer-N contains a net positive charge of +1, whereas the 8-mer from the C-terminus contains +4 this indicates that the ratio of basic residues to total residues is also important and a high ratio is favourable. Also, because the anticandidal activity of 8-mer, compared with 20-mer and 12-mer, was reduced, larger peptide(s) are required for considerable antifungal activity, and the 12-mer appears to be of the optimal length.

It is interesting to correlate and contrast our study with similar studies performed with bovine lactoferricin-derived and Hsn5-derived peptides. The study on the bovine lactoferricin B, a 25 amino acid residue peptide (FKCRRWQWRMCKLGAP-SITCVRRAF) [35] showed that peptide 2, a 10 amino acid residue peptide from the N-terminus of lactoferricin B (FKCR-RWQWRM), where four of the 10 residues are positively charged, possesses the strongest anticandidal activity. In fact, discarding the C-terminal region (15 amino acid residues, where four of the 15 residues are positively charged) increased the activity. The superior activity of peptide 2 thus appeared to be (at least in part) due to the high ratio of basic residues. In addition, this peptide up-regulated the polymorphonuclear function in cells and showed additive activity with other antifungal drugs (amphotericin B and miconazole).

Analysis of peptide fragments of Hsn-5 identified a 12 amino acid fragment of Hsn5 (amino acids 4–15 with net positive charge of 5), designated as P-113, as the smallest fragment that retained (but did not increase) anticandidal activity comparable with that of the parent compound [21]. P-113, designated here as Hsn5 12-mer, was selected to be used as a control peptide in the present study because of the same length as the MUC7 12-mer and containing a similar net positive charge (5 versus 6 respectively). Hsn5 12-mer (P-113) was shown in the original study [21] (and confirmed here) to retain the ability to form α -helical conformation in TFE solution, and by the helical wheel projection to form an amphipathic α helix. Additional study with the P-113-modified peptides indicated that the replacement of two or more of the basic residues of P-113 to uncharged residues resulted in a substantial loss of anticandidal activity. These results correlate well with our results obtained with the MUC7 12-mer variants.

Many structural parameters such as appropriate chain length, amino acid composition, peptide secondary structure, net positive charge and amphiphilicity were reported to have an influence on the activity and selectivity of antimicrobial peptides [13,36–39]. However, net positive charge and amphiphilicity were reported to be the most important factors governing the activity [13,37,40,41]. As pointed out previously [42], the positive charge of a peptide is consistent with its affinity for microbial membranes bearing negative charge, and amphipathy of a peptide is consistent with its capacity to intercalate into a membrane. MUC7 12-mer has a net positive charge of 6. Moreover, the helical-wheel projection of this peptide showed a good amphiphilic nature with well-defined and separate polar and hydrophobic faces. Decrease of the net positive charge by 2 lowered the cidal activity of the 12-mer. However, replacement of all six positively charged residues of the MUC7 12-mer (12-mer-3) resulted in a complete loss of activity against both fungi. Further experiments with the 12-mer-3 using fluorescence microscopy and membrane potential measurements indicated that the lack of activity is due to the loss of initial interaction of the peptide with the cell membrane and its subsequent uptake by the cells. Cysteine residues were all dispensable for the activity of the MUC7 12-mer. Although they seemed to be important for binding of MUC7 peptide to bacteria [25], their substitution by alanine did not affect the fungicidal activity of MUC7 12-mer.

Overall, the antifungal assays, fluorescent studies and membrane potential measurements suggest that both amphiphilic nature and high net positive charge of the MUC7 peptides are important determinants for the fungicidal activity, more specifically, for the initial interaction of the peptide with the fungal cells.

In summary, the results of the present study suggest that MUC7 12-mer peptide, a fragment of a naturally occurring human salivary mucin glycoprotein MUC7, may be an excellent candidate for development into a novel antifungal therapeutic agent. To lower the cost of antimicrobial peptides, it is of significance to minimize fragment length to the length that preserves or even exceeds the antimicrobial activity of the parent molecule. In that regard, the cost of chemical synthesis of the 12-mer peptide would be much reduced compared with that of MUC7 20-mer.

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