Novel α-Melanocyte Stimulating Hormone Peptide Analogues with High Candidacidal Activity

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α-Melanocyte stimulating hormone (α-MSH) is an endogenous linear tridecapeptide with potent antiinflammatory effects. We recently demonstrated that α-MSH and its C-terminal sequence Lys-Pro-Val (α-MSH (11−13)) have antimicrobial effects against two major and representative pathogens: *Staphylococcus aureus* and *Candida albicans*. In an attempt to improve the candidacidal activity of α-MSH and to better understand the peptide structure−antifungal activity relations, we designed and synthesized novel peptide analogues. Because previous data suggested that antimicrobial effects of α-MSH were receptor-mediated, we chose to focus on the sequence α-MSH (6−13), which contains the invariant core sequence His-Phe-Arg-Trp (6−9) that is important for binding to the known melanocortin receptors and also contains the sequence Lys-Pro-Val (11−13) that is known to be important for antimicrobial activity. In this structure−activity study, we discovered several compounds that have greater candidacidal activity than α-MSH. The peptide [D-Nal-7,Phe-12]-α-MSH (6−13) was the most potent of the analogues tested. The present results are very encouraging because they show the great potential of these peptides as a truly novel class of candidacidal compounds.

Introduction

The rapid emergence of microorganisms resistant to conventional antibiotics has hastened the search for new antimicrobial agents.[1−3] Natural antimicrobial peptides are promising candidates for treatment of resistant bacterial and fungal infections. They kill a broad spectrum of pathogens, and microorganisms resistant to them are uncommon.[4−11] Most of these peptides are cationic, but they differ considerably in other characteristics such as size and presence of disulfide bonds and structural motifs.[12,13] The mechanism of action of natural antimicrobial peptides has not been completely elucidated. Most of these peptides are believed to exert their antimicrobial activities through either formation of multimeric pores in the lipid bilayer of the cell membrane[14] or interaction with DNA or RNA after penetration into the cell.[15−17] Natural antimicrobial peptides are generally positively charged and therefore bind preferentially to negatively charged bacterial membranes rather than to mammalian cell membranes that have neutral charge.[18,19] A very important feature of antimicrobial peptides is that they rarely induce bacterial resistance.[14] Therefore, these molecules are promising candidates for a new class of antibiotics.[20]

α-Melanocyte stimulating hormone (α-MSH) (Figure 1) is an endogenous linear peptide derived from proopiomelanocortin (POMC). This peptide has potent antiinflammatory, antipyretic, and pigmentary effects.[21−23] Recognition and cloning of five melanocortin receptors (MC1R through MC5R),[24−29] which bind α-MSH and related melanocortins, have greatly improved understanding of peptide−target cell interactions and have opened novel possibilities for receptor-targeted therapies.[30] Antimicrobial influences of α-MSH were initially explored to test the idea that this established endogenous antiinflammatory agent could also be anti-infective.[31] Indeed, α-MSH is a very ancient peptide that appeared during the Paleozoic era, long before adaptive immunity appeared, and it has similarities with known natural antimicrobial peptides: it is produced by barrier epithelia[32−34] and it has a positive charge. α-MSH and its C-terminal tripeptide Lys-Pro-Val were discovered to have potent antimicrobial activity against two representative pathogens: *Staphylococcus aureus* and *Candida albicans*.[31] Evidence suggests that the antimicrobial influences of α-MSH are exerted through a unique mechanism substantially different from that of other natural antimicrobial peptides. Indeed, previous research suggests that the candidacidal effect of α-MSH is linked to the cAMP-inducing activity of the peptide: α-MSH increased cAMP production in *C. albicans* and the adenylyl cyclase inhibitor ddAdo partly reversed the candidacidal effect of the peptide.[31] It is remarkable that this mechanism of action in yeast mimics the influence of α-MSH in mammalian cells in which the peptide binds to G-protein-linked melanocortin receptors, acti-

α-MSH $Ac\text{-}Ser^{1}\text{-}Tyr^{2}\text{-}Ser^{3}\text{-}Met^{4}\text{-}Glu^{5}\text{-}His^{6}\text{-}Phe^{7}\text{-}Arg^{8}\text{-}Trp^{9}\text{-}Gly^{10}\text{-}Lys^{11}\text{-}Pro^{12}\text{-}Val^{13}\text{-}NH_2$

Figure 1. Structure of α-MSH.

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vates adenylyl cyclase, and increases cAMP.[21] Because both the cAMP inducer forskolin and the adenylyl cyclase inhibitor ddAdo alone reduced *Candida* colonies, it appears that cAMP is crucial in *C. albicans* viability and both marked increases and reductions in this mediator can reduce yeast viability.[31]

In the present study, the aims were to find α-MSH analogues with greater antimicrobial activity and to reach a better understanding of the peptide structure−antifungal activity relations through design, synthesis, and testing of novel peptide analogues in which several modifications were made. We chose the sequence α-MSH (6−13), which contains the invariant core sequence His-Phe-Arg-Trp (6−9) which is common to all melanocortin peptides and is important for binding to the known melanocortin receptors,[35] and the sequence Lys-Pro-Val (11−13), which is known to be relevant to antimicrobial activity.[31] We investigated whether substitution of Phe-7 with D-Phe-7 or D-Nal-7, known to result in superpotent α-MSH analogues in mammalian cells,[36,37] likewise increases the candidacidal activity of the peptide. On the basis of an alanine scan that indicated the importance of the Pro-12 residue for the candidacidal activity, we synthesized and evaluated analogues in which systematic replacements of this amino acid were made, alone or in combination with substitutions in position 7.

Experimental Procedures

Abbreviations. Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in the following reference: *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: Boc, *tert*-butyloxycarbonyl; *t*Bu, *tert*-butyl; cAMP, adenosine 3′,5′-cyclic-monophosphate; ddAdo, dideoxyadenosine; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Et_3SiH, triethylsilane; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); Pbf, 2,2,-4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; RIA, radioimmunoassay; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; Trt, triphenylmethyl (trityl). Amino acid symbols denote the L configuration unless indicated otherwise.

Materials. N^{α}-Fmoc-protected amino acids, HBTU, HOBt, and Wang resin were purchased from Advanced ChemTech (Louisville, KY). For the N^{α}-Fmoc-protected amino acids, the following side chain protecting groups were used: Arg(N^{g}-Pbf), Asp(*O*-*t*Bu), His(N^{im}-Trt), Trp(N^{in}-Boc), Glu(*O*-*t*Bu), Lys(Boc), Ser(*t*Bu), and Tyr(*t*Bu). Peptide synthesis solvents, reagents, and CH_3CN for HPLC were reagent grade, were acquired from commercial sources, and were used without further purification unless otherwise noted. FAB-MS experiments involved matrix-assisted laser desorption ionization (MALDI). The purity of the finished peptides was checked by analytical RP-HPLC using a Shimadzu model CL-10AD VP system with a built-in diode array detector. In all cases, the purity of the finished peptides was greater than 95% as determined by these methods.

General Method for Peptide Synthesis and Purification. All peptides were synthesized by the solid-phase method of peptide synthesis and purified by RP-HPLC. The peptides were each synthesized on 0.15 g of Wang resin (substitution 0.7 mmol/g) by manual methods and by Advanced ChemTech automated peptide synthesizer, 348 Ω, using N^{α}-Fmoc chemistry and an orthogonal side chain protection strategy. The entire synthesis was performed under argon. The resin was first swollen in DCM/DMF (1:1) for 2 h, and the following amino acids were then added to the growing peptide chain by stepwise addition of N^{α}-Fmoc-Val-OH, N^{α}-Fmoc-Pro-OH, N^{α}-Fmoc-Lys(Boc)-OH, N^{α}-Fmoc-Gly-OH, N^{α}-Fmoc-Trp(Boc)-OH, N^{α}-Fmoc-Arg(N^{g}-Pbf)-OH, N^{α}-Fmoc-Phe-OH, and N^{α}-Fmoc-His(N^{im}-Trt)-OH, using standard solid-phase methods. Each coupling reaction was achieved using a 3-fold excess of each of the amino acids, HBTU, and HOBt in the presence of a 6-fold excess of DIPEA for 1 h. Deprotection of the N^{α}-Fmoc group was carried out by treating the protected peptide resin with 25% piperidine solution in DMF (1 × 4 mL, 20 min). After each coupling and deprotection, the peptide resin was washed with DMF (3 × 4 mL), DCM (3 × 50 mL), and again with DMF. The peptide sequences were thus assembled by alternate cycles of coupling and deprotection. After coupling of the N-terminal amino acid, the N-terminal Fmoc group was deblocked as described above and the peptide resin was thoroughly washed with DCM (4 × 25 mL) and dried under argon to yield dried peptide resin.

The peptide resin was then cleaved by treating with 4 mL of a solution of Et_3SiH (5%), water (5%), and *p*-thiocresol/*p*-cresol (0.1%, 1:1) in TFA with shaking at room temperature for 3 h. The resin was then removed from the solution by filtration, and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether. Centrifugation at 1500*g* for 3 min followed by decantation of the supernatant ether and air-drying of the residue yielded the crude peptide as a white to pale-beige amorphous solid.

Final peptide purification was achieved using a preparative RP-HPLC Vydac C18 (218TP1520, 15 μm). The peptide samples were injected onto the column at a concentration of 20−30 mg/mL in 20% aqueous CH_3CN and were eluted with a CH_3CN gradient (10−90%) over 40 min at a flow rate of 15.0 mL/min with a constant concentration of TFA (0.1% v/v). The separations were monitored at 230 and 280 nm and integrated with a Shimadzu diode array detector model SPD-M10A VP dual-wavelength absorbance detector model UV-D. Fractions corresponding to the major peak were collected, pooled, and lyophilized to yield the final peptides as pure (>95%) white solids. Amino acid analyses were carried out using a Pico-Tag workstation. Lyophilized samples of peptides (50−1000 pmol) were hydrolyzed in heat-treated borosilicate tubes (4 mm × 50 mm) using the Pico-Tag workstation (Waters-Millipore, Waltham, MA) for 1 h at 150 °C with 200 mL of 6 N HCl containing 1% phenol. A Pico-Tag column (3.9 mm × 15 mm) was employed to separate the amino acid derivatives. The analytical data and the amino acid analysis for each compound are presented in Tables 1 and 2.

Organism and Culture Conditions. Five clinical isolates of *Candida albicans* and the ATCC 24433 strain were obtained from the collection of the laboratory of Microbiology, Ospedale Maggiore di Milano. *Candida* cells were maintained on Sabouraud's agar slants and periodically were transferred to Sabouraud's agar plates and incubated for 48 h at 28 °C. To prepare stationary growth-phase yeast, a colony was taken from the agar plate and transferred into 5 mL of Sabouraud dextrose broth and incubated for 48 h at 32 °C. Cells were centrifuged at 1000*g* for 10 min, and the pellet was washed twice with distilled water. Cells were counted and suspended in distilled water to obtain 10^7 yeast cells/mL. Viability, determined by the exclusion of 0.01% methylene blue, remained greater than 98%.

Colony Formation. Tubes containing 1×10^6 *Candida albicans* in 100 μL of distilled water were treated with 100 μL of each peptide (10^{-4} M final concentrations). Control tubes received 100 μL of distilled water. All the tests were run in triplicate. After 2 h of incubation at 37 °C, yeast suspensions from each vial were diluted with distilled water to obtain approximately 100 organisms/mL. A 1 mL aliquot from each tube was dispensed on blood agar plates and was incubated for 48 h at 37 °C. Colony-forming units (CFU) were then counted. Organism viability was estimated from the number of CFU.

Table 1. Physicochemical Properties of the Peptides

peptide	R_f [a]	MW	MS, *m/z*
1	4.69	1025.23	1026.35
2	5.08	1075.30	1076.50
3	4.28	968.14	969.45
4	4.31	999.14	1000.70
5	4.45	997.18	998.40
6	4.69	1025.23	1026.30
7	4.65	1039.26	1040.80
8	4.31	999.14	1000.45
9	4.60	1049.25	1050.30
10	4.71	1049.25	1050.40
11	4.08	985.17	986.20
12	4.45	1035.20	1036.30
13	4.21	1015.19	1016.45
14	4.60	1065.25	1066.20
15	5.11	1075.30	1076.20
16	5.15	1075.30	1076.35
17	5.10	1075.30	1076.30
18	5.08	1075.30	1076.30
19	6.36	1125.35	1126.30
20	6.21	1125.35	1126.35
21	4.47	1043.20	1044.30
22	4.45	1043.20	1044.45
23	4.48	1043.20	1044.40
24	4.81	1093.26	1094.30
25	4.51	1057.23	1058.40
26	4.92	1107.25	1108.60
27	4.77	1056.30	1057.70
28	5.12	1106.33	1106.60

[a] HPLC: $k' = [(\text{peptide retention time}) - (\text{solvent retention time})]/(\text{solvent retention time})$ in a solvent system of 10% CH_3CN in 0.1% TFA and a gradient to 90% CH_3CN over 40 min. An analytical Vydac C_{18} column was used with a flow rate of 1 mL/min.

Table 2. Amino Acid Analysis of the Peptides[a]

	fraction of amino acid						
peptide	His	Yaa[a]	Arg	Gly	Xaa[a]	Xbb[a]	Xcc[a]
1	0.99	0.95	0.93	0.94	0.98	0.93	1.02
2	0.94	0.92	0.95	1.01	0.91	0.98	0.93
3	0.98	0.98	0.90	0.96	0.91	0.94	0.96
4	0.99	1.02	0.95	0.99	0.98	0.98	0.96
5	1.01	0.98	0.98	0.97	0.90	0.92	1.03
6	0.98	0.95	0.99	0.96	1.03	1.05	0.97
7	0.99	0.94	0.93	0.97	0.92	0.91	0.94
8	0.93	0.98	0.97	0.99	0.89	0.91	0.94
9	0.92	0.91	1.03	1.01	0.98	0.92	0.96
10	1.01	0.93	0.87	0.92	0.91	0.96	0.98
11	0.98	0.89	1.03	0.97	0.92	0.97	0.93
12	0.95	0.95	0.96	0.89	1.08	0.98	0.91
13	0.91	0.98	0.96	0.98	0.89	0.96	1.02
14	1.03	1.02	0.99	0.98	0.84	0.91	0.98
15	0.93	0.98	0.97	0.99	0.93	0.97	1.01
16	0.94	0.95	0.94	1.01	0.83	0.98	0.98
17	0.97	0.92	1.01	0.99	0.96	1.03	0.94
18	0.93	1.03	0.94	0.94	0.96	0.97	0.97
19	0.97	0.94	0.97	0.95	0.98	0.96	0.97
20	0.97	0.98	0.99	0.99	0.89	0.96	1.03
21	0.97	0.91	1.01	0.93	0.84	0.91	0.93
22	0.92	0.93	0.92	0.92	0.96	0.97	0.93
23	1.06	0.89	0.97	1.01	0.91	0.89	0.97
24	0.95	0.95	0.96	0.98	0.97	1.05	0.88
25	0.95	0.98	0.96	0.98	0.98	1.10	1.02
26	0.91	1.02	0.99	0.98	1.03	0.90	0.98
27	1.03	0.98	0.97	0.99	0.97	1.01	1.01
28	0.93	0.95	0.94	1.01	0.96	0.98	0.98

[a] Trp was not well determined because of decomposition under these conditions. [b] Value for the corresponding amino acid (see Table 3).

Statistical Analysis. One-way analysis of variance and Student's *t* test were used to analyze the data. Probability values less than 0.05 were considered significant.

Results and Discussion

We designed and synthesized α-MSH peptide analogues with different residues in the core and in the C-terminal sequences and tested their candidacidal activity. The criteria adopted were similar to those used to design analogues active in mammalian cells.[38] Indeed, the capacity of the parent α-MSH peptide to induce cAMP in *Candida* cells[31] suggested the presence of one or more melanocortin receptor(s). Although the actual existence of such receptor(s) in yeast has not been established, we examined the effects of amino acid substitutions based on knowledge that receptor binding and transducing properties of melanocortins depend on separate structural and conformational characteristics.[21] For example, Phe-7 plays a key role in receptor binding whereas Lys-11 and Pro-12 are more important for receptor stimulation.[21]

Previous research suggested that the candidacidal effect of α-MSH (1−13) does not depend on the N-terminal residues, which are also less important for peptide activity in mammalian cells.[38,39] Further, a smaller amino acid sequence would be more suitable for clinical antifungal use. Therefore, we focused on the fragment α-MSH (6−13) that contains the invariant core sequence His-Phe-Arg-Trp (6−9), which is crucial for binding to melanocortin receptors,[35] and on the sequence Lys-Pro-Val (11−13) relevant for both receptor activation[21] and antimicrobial activity.[31]

Substitutions in position 7 of the core sequence that are known to alter potency of melanocortins in mammalian cells were explored with regard to influences on candidacidal activity. Indeed, substitution of Phe-7 with D-Phe-7 or D-Nal-7 markedly enhances potency of melanocortins.[37,40] (Nle-4-D-Phe-7)-α-MSH[36] has been the most used of α-MSH analogues in vivo and in vitro for 2 decades. Candidacidal activity of peptide **1** (D-Phe-7) was slightly increased relative to the lead molecule α-MSH (6−13), but variability across experiments was very great (Table 3). Such substitution did not alter substantially the candidacidal effect of the peptide even when it was associated with specific replacements in the C-terminal region (see below). Therefore, the increased activity of melanocortins linked to D-Phe-7 substitution appears to be restricted to mammalian cells. Conversely, D-Nal-7 substitution (peptide **2**) caused a remarkable and very consistent increase in activity (95.3% ± 7.7 vs 59.4% ± 15.5 of α-MSH (6−13)). Because D-Nal-7 is known to increase the affinity of α-MSH for MC4R,[39] there may be similarities between this melanocortin receptor and the yeast receptor.

The next step consisted of an alanine scan to determine the contribution of each amino acid in the Lys-Pro-Val sequence to candidacidal activity. Data showed that Lys-11 and Pro-12 are essential in such activity, whereas Val-13 is not. Lys-11 and especially Pro-12 substitutions nearly abolished antifungal activity (peptides **3** and **4**). Replacement of the Val residue in position 13 was possible without loss of activity (peptide **5**). Replacing L-Val-13 with either its D isomer (peptide **6)** or Leu (peptide **7**) did not significantly change the candidacidal activity, confirming that this residue is not crucial.

Previous structure−activity studies on the α-MSH (11−13) sequence disclosed the significance of Pro-12

Table 3. Candidacidal Activity of the Peptides (100 μM)

peptide	structure	% inhibition	SD
α-MSH (6−13)	H-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	59.4	15.5
1	H-His-D-***Phe***-Arg-Trp-Gly-Lys-Pro-Val-NH_2	80.5	27.0
2	H-His-D-***Nal***-Arg-Trp-Gly-Lys-Pro-Val-NH_2	95.3	7.7
3	H-His-Phe-Arg-Trp-Gly-***Ala***-Pro-Val-NH_2	26.2	29.1
4	H-His-Phe-Arg-Trp-Gly-Lys-***Ala***-Val-NH_2	12.8	18.1
5	H-His-Phe-Arg-Trp-Gly-Lys-Pro-***Ala***-NH_2	68.4	31.5
6	H-His-Phe-Arg-Trp-Gly-Lys-Pro-D-***Val***-NH_2	82.5	26.8
7	H-His-Phe-Arg-Trp-Gly-Lys-Pro-***Leu***-NH_2	87.4	22.7
8	H-His-Phe-Arg-Trp-Gly-Lys-D-***Ala***-Val-NH_2	46.1	30.0
9	H-His-D-***Nal***-Arg-Trp-Gly-Lys-***Ala***-Val-NH_2	28.0	24.5
10	H-His-D-***Na***l-Arg-Trp-Gly-Lys-D-***Ala***-Val-NH_2	69.2	27.1
11	H-His-Phe-Arg-Trp-Gly-Lys-***Gly***-Val-NH_2	41.8	27.9
12	H-His-D-***Nal***-Arg-Trp-Gly-Lys-***Gly***-Val-NH_2	36.2	24.2
13	H-His-Phe-Arg-Trp-Gly-Lys-***Ser***-Val-NH_2	32.3	25.5
14	H-His-D-***Nal***-Arg-Trp-Gly-Lys-***Ser***-Val-NH_2	73.9	25.0
15	H-His-Phe-Arg-Trp-Gly-Lys-***Phe***-Val-NH_2	90.8	9.3
16	H-His-Phe-Arg-Trp-Gly-Lys-D-***Phe***-Val-NH_2	97.5	4.2
17	H-His-D-***Phe***-Arg-Trp-Gly-Lys-***Phe***-Val-NH_2	89.6	14.5
18	H-His-D-***Phe***-Arg-Trp-Gly-Lys-D-***Phe***-Val-NH_2	77.7	31.5
19	H-His-D-***Nal***-Arg-Trp-Gly-Lys-***Phe***-Val-NH_2	99.7	0.4
20	H-His-D-***Nal***-Arg-Trp-Gly-Lys-D-***Phe***-Val-NH_2	67.7	29.1
21	H-His-Phe-Arg-Trp-Gly-Lys-***Asp***-Val-NH_2	5.9	9.1
22	H-His-Phe-Arg-Trp-Gly-Lys-D-***Asp***-Val-NH_2	15.7	15.6
23	H-His-D-***Phe***-Arg-Trp-Gly-Lys-***Asp***-Val-NH_2	3.7	4.9
24	H-His-D-***Nal***-Arg-Trp-Gly-Lys-***Asp***-Val-NH_2	16.8	22.3
25	H-His-Phe-Arg-Trp-Gly-Lys-***Glu***-Val-NH_2	11.2	12.7
26	H-His-D-***Nal***-Arg-Trp-Gly-Lys-***Glu***-Val-NH_2	32.3	25.6
27	H-His-Phe-Arg-Trp-Gly-Lys-***Lys***-Val-NH_2	41.0	26.9
28	H-His-D-***Nal***-Arg-Trp-Gly-Lys-***Lys***-Val-NH_2	83.8	26.9

to the antiinflammatory influence of the tripeptide.[41] Indeed, substitution of Pro-12 with its D isomer abolished the antiinflammatory effect.[41] Subsequently, research on C-terminally modified analogues of α-MSH confirmed the important role of Pro-12 for binding and activity at the MC1 receptor.[42] As stated above, the alanine scan also demonstrated the importance of Pro-12 with regard to candidacidal influences. Therefore, we explored further the significance of Pro-12, an uncharged amino acid, to the antimicrobial effect by replacing it with several amino acids with different physicochemical properties.

Peptides **8**, **11**, and **13**, containing the uncharged and nonlipophilic residues D-Ala, Gly, and Ser in position 12, had lesser activity than α-MSH (6−13). Activity was only partly restored by association of D-Nal-7 in peptides **10** and **14**.

Peptides **15**, **17**, and **19**, in which Pro-12 was replaced with the hydrophobic amino acid Phe, showed pronounced candidacidal activity. Activity was further enhanced when Phe-12 was associated with D-Nal-7 in peptide **19**. This peptide killed 100% of the *C. albicans* on repeated experiments (99.7% ± 0.4) and is therefore the most promising compound of the present series. The substitution D-Phe-12 enhanced the antifungal activity only in combination with the natural Phe residue in position 7 (peptide **16**). Peptides **18** and **20** with a D-Phe residue in position 12 associated with either D-Phe7 or D-Nal-7 showed similar candidacidal activity relative to the lead compound α-MSH (6−13). This suggests subtle relations in receptor binding affinity.

Enhanced candidacidal activity of the Phe-12-substituted peptides was the most distinctive feature relative to effects of similar analogues in mammalian cells. Indeed, when Pro-12 in the α-MSH (1−13) sequence was replaced with Phe-12, peptide potency at rodent melanocortin receptors decreased.[42] Activity was restored by concurrent D-Phe-7 replacement.[42] In yeast cells, concomitant D-Phe-7 substitution did not alter potency of these peptides. Peptide **17** [D-Phe-7,Phe-12] was as effective as peptide **15** [Phe-7,Phe-12], and both were much more potent than the lead nonsubstituted compound.

A very interesting observation was that peptides **21**−**26**, which contain Asp-12 or Glu-12, two amino acids with a negative charge in the side chain, did not have candidacidal activity. Activity was not restored even if these substitutions were associated with D-Nal-7. These observations suggest that, much as for other antimicrobial peptides,[19] a negative charge in the C-terminal region prevents peptide−microbial interaction and reduces antimicrobial activity.

Structural parameters such as net positive charge, hydrophobicity, peptide helicity, hydrophobic moment, and the size of peptide influence activity and selectivity of membrane-active peptides.[43] Among structural parameters, net positive charge and hydrophobicity appear to be the most relevant factors with regard to activity and selectivity. A good example is provided by substitutions in the decapeptide KLS, a potent antifungal peptide recently discovered.[19] The net positive charge was positively correlated with peptide activity, whereas increases in hydrophobicity progressively reduced antifungal effects. α-MSH analogue **19**, in which hydrophobicity was increased but the net positive charge was not altered, showed remarkable candidacidal activity. Conversely, Lys substitution in position 12 in peptides **27** and **28** did not enhance activity relative to the lead compound or the D-Nal-7-substituted peptide **2** despite the increase in the net positive charge. These observations further support the idea of a unique mechanism of action of α-MSH peptides that is substantially different from that of most antimicrobial agents that cause direct damage to the microbial membrane.

Conclusions

Results indicate that substitutions in the α-MSH (6−13) amino acid sequence can either enhance or reduce candidacidal influences of the peptide. Peptides **15**−**19** reduced viability of *Candida albicans* more effectively than α-MSH (6−13). It is of interest that peptides **15**, **16**, and **19** were even more effective than equimolar concentrations of the complete α-MSH (1−13) peptide. Peptide **19** killed almost 100% of the *Candida* cells over repeated experiments and is the most promising compound in the current series.

α-MSH peptides have been extensively used in preclinical studies and in vitro, and they have little or no toxicity even when given in massive amounts. Further, certain additional influences of the peptides in modulation of host responses, including antipyretic and anti-inflammatory effects,[22] could be helpful during microbial invasion. Therefore, at variance with other natural antibiotics that may be toxic for human cells, complete selectivity for microbial cells does not appear to be an absolute requirement for α-MSH peptides.

The present results are very encouraging in that they show the great potential of α-MSH analogues as a truly novel class of candidacidal compounds.

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