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Hb40-61a: Novel analogues help expanding the knowledge on chemistry, properties and candidacidal action of this bovine α-hemoglobin-derived peptide☆

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ABSTRACT

This study expands the knowledge on chemical synthesis and properties of *Hb40-61a* as well as provides results of the first steps given towards knowing how it kills *Candida* cells. For the first time, this peptide, its all-D analogue (*D-Hb40-61a*) and its fluorescently labeled analogue (*FAM-Hb40-61a*) were successfully assembled on resin at 60 °C using conventional heating in all steps. Purified and characterized, these peptides exhibited very low toxicity on human erythrocytes. *Hb40-61a* and *D-Hb40-61a* were equally active against *Candida* strains, ruling out sterically specific interactions on their working mechanism. Cell permeabilization assays confirmed progressive damage of the yeast plasma membrane with increasing concentrations of *Hb40-61a*. While experiment using the fluorescent probe $DiBAC_4(5)$ revealed that this synthetic hemocidin alters the yeast plasma membrane potential, test employing DPH indicated that *Hb40-61a* might affect its dynamics. Exposure of the yeast cells to *FAM-Hb40-61a* showed that the peptide accumulates in the cell membrane at the ½ MIC, but stains about 97% of the cells at the MIC. Such effect is salt-dependent and partially energy-dependent. These new findings indicate that the central target of *Hb40-61a* in *Candida* cells is the plasma membrane and that this synthetic hemocidin should be considered as a potential candidacidal for topic uses.

1. Introduction

Invasive candidiasis has become a worldwide concern as studies from the last two decades have shown that *Candida* species are the fourth most common cause of nosocomial bloodstream infection [1,2]. In fact, its mortality rate is now about 50% [3–5] and there is a global shift in epidemiology towards non-*albicans Candida* species [6]. As to candidemia, this is the major problem in tertiary-care hospitals because it causes morbidity and mortality in patients, especially in the immunocompromised ones [7]. Sadly, in Brazil, candidemia rates are 2–8 times higher than those documented in the USA and Europe [8,9].

The commercial antifungals are polyenes (amphotericin B and nystatin), azoles (fluconazole, itraconazole and ketoconazole), flucytosine and echinocandins (caspofungin, micafungin and anidulafungin). These compounds are no longer fully protecting people from fungal infections due to their high toxicity (mainly polyenes) and possibility to cause fungal resistance (mainly polyenes, azoles or echinocandins) [10–14]. In addition, their indiscriminate use has been the main reason of the current and growing number of organisms tolerant to them in therapeutic doses, a situation that represents a serious risk mainly to immunocompromised people [7].

Antimicrobial peptides (AMPs) are promising pharmacological compounds and rich sources of knowledge owing to their potential as inhibitors of the growth or killers of a variety of microorganisms as well as their antitumor and immunomodulator activities. The AMPs with antifungal and fungicidal activities can be more specific, but share the following properties with the other AMPs: they act quickly [15], they are able to kill strains resistant to the commercial antibiotics and they do not easily induce microorganism resistance [16]. Most of these molecules have a short chain peptide (12 to 50 amino acids), net positive charge (+2 to +9) and amphipathic structure [17] containing about 50% of hydrophobic residues [18]; they can acquire α-helix, β-sheet, extended or loop structures [19]. All these features are responsible for the initial stages of their mechanisms of action which are (i) the electrostatic attraction to negatively charged phospholipids and (ii) hydrophobicity-assisted insertion and disturbance of the membrane [20].

The expression hemocidin (*hemo*globin microbi*cid*al peptides) was first defined by Mak and colleagues as a way of describing AMPs that

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emerge *in vivo* or *in vitro* from heme-binding proteins (hemoglobin, myoglobin and cytochrome c) [21–23]. In 1999, we reported the first hemocidin with antimicrobial activity generated in the gut contents of the cattle tick *Boophilus microplus* by bovine α-hemoglobin proteolysis: fragments 33–61, named *Hb33-61*, whose amino acid sequence is F^{33}LSFPTTKTYFPHFDLSHGSAQVKGHGAK^{61} [24]. Subsequently, other hemocidins were described to be produced *in vitro* (for instance, resulting from heme-binding protein digestion in the presence of cyanogen bromide or from bovine hemoglobin proteolysis catalyzed by pepsin) or in vivo (found in gut contents of the rabbit tick *Ornithodorus moubata* or in human placental blood or, again, in the gut contents of the cattle tick *B. microplus*) [21,25–40].

Recognizing that C-terminal amidation increases peptide resistance to proteolysis and may potentiate its antimicrobial activity [24], in 2005 we reported the synthesis and study of the amidated analogue of *Hb33-61* F^{33}LSFPTTKTYFPHFDLSHGSAQVKGHGAK-NH_2 (*Hb33-61a*). The purified synthetic hemocidin was four-fold more potent than *Hb33-61* in inhibiting the growth of *Candida albicans MDM8* cells and, when in 200 μM sodium dodecyl sulfate (SDS) micelles, it folded very close to the structure acquired by fragments 33–61 while as part of the native bovine hemoglobin α-chain [41]. Having observed that *Hb33-61* and *Hb33-61a* also presented low hemolysis and knowing that reduction of molecular size is prized in pharmaceutical development [42], we then examined the structure–activity relationship of *Hb33-61a* using N- and C-terminally truncated analogues and proposed that *Hb40-61a* (K^{40}TYFPHFDLSHGSAQVKGHGAK^{61}-NH_2) could be its minimal active portion. In fact, *Hb33-61a* and *Hb40-61a* presented the same MIC (3.12–6.25 μM), causing the death of *C. albicans MDM8*, as it was shown to contain the main structural elements found in *Hb33-61a*: a β-turn in N-terminus (portion Lys^{40}-Phe^{43}) and a β-turn (portion Ser^{49}-Ser^{52}) followed by an α-helix (portion Ala^{53}-Lys^{61}) in the C-terminus, which led us to hypothesize that these two structured regions, by fluctuating independently in the lipid environment, could act in a coordinated fashion to permeabilize the yeast plasma membrane [43]. The mean hydrophobicity result for *Hb40-61a*, calculated according to Kyte and Doolitle (http://www.bbcm.units.it/~tossi/HydroCalc/HydroMCalc.html) was −1.1 [43], confirming its, although not perfect, amphipathic nature.

In those studies [24,40,42,43], *Hb33-61*, *Hb33-61a* and their truncated analogues, including *Hb40-61a*, were synthesized by stepwise solid-phase syntheses (SPPS) using Boc chemistry (use of dichloromethane and HF) and traditional protocols (room temperature in all synthetic steps). The crude materials obtained were quite heterogeneous and contained low amounts of the desired peptides, thus the final yields were very low, a limitation to be defeated.

In an attempt to expand the knowledge on chemical synthesis and properties of *Hb40-61a* as well as to give the first steps towards understanding how this synthetic hemocidin kills *Candida* cells, we performed the present study that comprises: (i) the first solid-phase syntheses at 60 °C using conventional heating in all steps of *Hb40-61a*, *D-Hb40-61a* (its all-D analogue) and *FAM-Hb40-61a* (its fluorescently labeled version); (ii) a comparative evaluation of the anticandidal and hemolytic activities of these peptides; and (iii) experiments designed to examine its properties and investigate at a general level how *Hb40-61a* kills *Candida* cells.

2. Materials and methods

2.1. Peptide synthesis, purification and chemical characterization

The peptides were manually synthesized using the Fmoc-chemistry and protocols developed in our laboratories for stepwise solid-phase synthesis (SPPS) at 60 °C in all steps using conventional heating [44–46]. Peptide assembly started from RINK-amide or CLEAR amide resin. Fmoc removal was done in 20% piperidine/DMF for 7 min (twice, when needed). Fmoc-amino acids (2.5-fold excess) were coupled (and recoupled, when needed) for 30 min in the presence of DIC/HOBt (1:1, equimolar to the Fmoc-amino acid) or TBTU (equimolar to the Fmoc-amino acid)/DIPEA (7.5-fold excess) in 20% DMSO/NMP or DMF as solvent systems, respectively. Alternate washings of the peptide resins employed 20% DMSO/NMP (for reactions carried-out in this solvent mixture) or DMF (for reactions performed in this solvent), MeOH (for RINK resin) or isopropanol (for CLEAR). The coupling of the 5(6)-carboxyfluorescein (*FAM*) to the resulting fully protected peptide-resins was also performed at 60 °C using 5 equiv. of *FAM*, 5 equiv. of benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) and 5 equiv. of HOBt in 20% DMSO/NMP for 2 h [46,47]. Ninhydrin test was used to monitor each deprotection or coupling reaction.

Full deprotection and simultaneous cleavage of the peptide from resin were carried out in the presence of TFA (95%), water (2.5%) and TIS (2.5%). The free peptide was precipitated in diisopropyl ether and extracted with 0.1% TFA/water or 60% ACN/0.1% TFA/water. The resulting solution was lyophilized to give the solid crude material.

Peptide purification by reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters system composed by a Delta 600 Pump, a 2487 Dual Absorbance detector, a 3725i-119 Rheodyne injector and a 600 Controller (Millford/MA, USA) and a Kipp & Zonen SE 124 register connected to a Grace-Vydac C_{18} column (10 μm, 300 Å, 2.2 × 25.0 cm; Albany/OR, USA), a flow rate of 9 mL/min, and a detection wavelength of 220 nm. The purifications required two steps [48]: (1) triethylammonium phosphate (TEAP)/water at pH 2.25 as solvent A, 50% ACN/TEAP as solvent B; (2) 0.1% TFA/water as solvent A, 60% ACN/A as solvent B. Elution was achieved using appropriate linear gradients.

Peptide identity was confirmed by liquid chromatography coupled to electrospray ionization mass spectrometry (LC-MS). LC was performed on a Shimadzu system (Kyoto, Japan) composed by a TGU-$20A_3$ degasser, two LC-20AD pumps, a 8125 Rheodyne and a CTO20A column oven connected to a C_{18} pre-column (4.6 mm, 12 nm, 5 × 2 mm) Shimpack GVP-ODS and a C_{18} column (4.6 mm, 12 nm, 150 × 2 mm). MS used ESI positive mode and an ion trap Bruker instrument (AmaZonX, Bruker Daltonics, Fahrenheitstrasse, Germany).

Peptide content, required for exact determination of peptide concentration in biological assays, was given by amino acid analysis, which was done on a Dionex BioLC® Chromatography system (Sunnyvale/CA, USA) composed by an AS40 automatic sampler, a GS50 quaternary pump, an LC25 column oven, a 2 × 250 mm PA10 AminoPac ion-exchange column and an ED50 electrochemical detector. Therefore, prior to the biological assays, each peptide preparation had the precise peptide concentration determined.

2.2. Fungal strains, antifungal and fungicidal activity assays

C. albicans (ATCC 90028), *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019). The strains were cultured overnight in an incubator shaker at 30 °C in Sabouraud Dextrose Broth (DB, Sparks/MD, USA. The cells were diluted 1:100 in the same medium and incubated for 4 h at 30 °C to enrich the population of exponentially growing cells and reach the log-phase. The cells were centrifuged for 5 min at 8000 *g* and washed three times with PDB or LB medium.

The antimicrobial activity was evaluated by liquid growth-inhibition assay as described by us [41,43]. Mellitin was used as control. The MIC values for the peptides were expressed as the intervals of concentrations [a]–[b], where [a] is the highest concentration tested at which the microorganism was growing and [b] is the lowest concentration that inhibits the visible microbial growth. Peptide concentrations were in the range of 0.19–100 μM. Three independent experiments were performed.

For the candidacidal assays, *C. albicans* ATCC 90028, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 cells were incubated for 48 h in the presence of the peptides or water (control) under the conditions