Antifungal activity of the non cytotoxic human peptide hepcidin 20 against fluconazole resistant Candida glabrata in human vaginal fluid

Running title: Activity of hepcidin 20 on Candida glabrata

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Vaginal infections due to *Candida glabrata* are difficult to eradicate, due to the scarce susceptibility to azoles of this species. Previous studies have shown that the human cationic peptide hepcidin 20 (Hep-20) exerts fungicidal activity in sodium phosphate buffer against a panel of *C. glabrata* clinical isolates with different susceptibility to fluconazole. In addition, the activity of the peptide was potentiated under acidic condition, suggesting a potential application in the topical treatment of vaginal infections. To investigate whether the peptide activity could be maintained in biological fluids, in this study the antifungal activity of Hep-20 was evaluated by killing assay in (i) a vaginal fluid simulant (VFS), and in (ii) a human vaginal fluid (HVF) collected from three healthy donors. The results obtained indicated that the activity of the peptide was maintained in VFS and HVF supplemented with EDTA. Interestingly, the fungicidal activity of Hep-20 was enhanced in HVF, in comparison to that observed in VFS, with a minimal fungicidal concentration of 25 μM for all donors. No cytotoxic effect on human cells was exerted by Hep-20 at concentrations ranging from 6.25 to 100 μM, as shown by XTT reduction assay and propidium iodide staining. An indirect evidence of Hep-20 stability was also obtained from co-incubation experiments of the peptide with HVF at 37°C for 90 minutes and 24 hours. Collectively, these results indicate that this peptide should be further studied as a potential novel therapeutic agent for the topical treatment of vaginal *C. glabrata* infections.
INTRODUCTION

In the last decade there have been increasing reports of vaginitis due to non-Candida albicans species, which now accounts for 20% of Candida vaginal infections (1). Candida glabrata has emerged as an important fungal pathogen, and its clinical relevance is partially due to its intrinsic or rapidly acquired resistance toazole antifungal drugs, such as fluconazole, commonly used in the treatment of mucosal infections (1, 2). It has been postulated that the rise in C. glabrata prevalence from recurrent vulvovaginal candidosis observed in the last few decades is related to the widespread use of fluconazole, to which this species is less sensitive than C. albicans and other non-albicans Candida species (1, 2). Recent reports have revealed an increase of C. glabrata clinical isolates that display resistance to not only azoles, but also to echinocandins (3, 4). This aspect complicates the management of C. glabrata vaginal infections, which as a result are difficult to eradicate and often end up in recurrent episodes (1).

Considering the limitations of the currently available antifungals, it has become mandatory to identify new classes of antimicrobial compounds to provide an effective antifungal strategy for a broad range of fungal pathogens, including C. glabrata.

In this regard, natural anti-infective agents, such as antimicrobial peptides (AMPs), represent a promising approach due to their wide-spectrum activity, rapid mode of action and microbicidal effect on multi resistant microorganisms (5). Interactions between AMPs and microbial surface play a crucial role in determining the activity of membrane-active AMPs. The positive charge of the vast majority of AMPs allows the initial interaction with the negatively charged cell wall, and later with negatively charged phospholipids in the inner membrane. Following interaction with the lipid bilayer, most AMPs cause displacement of lipids, alteration of membrane structure and membrane perforation (5).

Structurally different AMPs, obtained from different organisms including amphibians, plants and mammals have been proven to exert antifungal activity versus clinically relevant Candida
species (6). However, C. glabrata is shown to be scarcely susceptible to a wide spectrum of AMPs from different derivation; among these, histatin, cathelecidins, defensins and magainin (7-10). We have previously shown that the human liver-derived peptide hepcidin 20 (Hep-20) exerts antifungal activity against C. glabrata clinical isolates characterized by different susceptibility to fluconazole (11). In addition, the fungicidal effect exerted by Hep-20 on C. glabrata isolates in sodium phosphate buffer (SPB) was potentiated under acidic conditions (pH 5.0) (11), according to a proposed mechanism by which at acid pH protonated basic residues of Hep-20 may facilitate the interaction with negatively charged fungal surfaces, as already reported for other peptides (12, 13). The enhanced fungicidal activity of Hep-20 observed under acidic conditions suggested that this peptide could be used as potential therapeutic agent in the topical treatment of C. glabrata vaginal infection. However, a direct application of in vitro promising AMPs as novel therapeutic agents has so far been held back by several limitations, including a significant reduction of their antimicrobial activity in the presence of biological fluids due to physiological concentration of salts, such as K⁺, Na⁺, Mg²⁺ and Ca²⁺ (14-17). In addition, clinical trials performed so far on AMPs have been restricted to topical applications, due to their poor bioavailability associated with host proteases susceptibility and unknown toxicity profiles (18). Therefore, to evaluate the potential application of Hep-20 in the topical treatment of C. glabrata vaginitis, the peptide activity was evaluated in i) a vaginal fluid simulant (VFS), resembling human secretion and ii) in human vaginal fluid (HVF), collected from three healthy donors. In addition, despite the peptide was previously shown to produce no significant effect on human erythrocytes (11) and on erythroleukemia K562 human cells (19), we further investigated Hep-20 toxicity versus human peripheral blood mononuclear cells (PBMCs) and an epithelial cell line (A459) by XTT reduction assay and propidium iodide (PI) staining. Indirect evidence of the peptide stability was also obtained by co-incubating Hep-20 in the presence of HVF.
2. MATERIALS AND METHODS

2.1 Yeast strain and growth media
A *Candida glabrata* clinical isolate resistant to fluconazole (BPY44, MIC 256 μg/ml) was used in this study (11). BPY44 was grown in Sabouraud broth (Liofilchem S.r.l., TE, Italy) at 30°C for 18 hours. The strain was maintained on Sabouraud agar (Liofilchem S.r.l.) for the duration of the study.

2.2 Antimicrobial peptide
Synthetic Hep-20 was purchased from Peptide Specialty Laboratories GmbH (Heidelberg, Germany). The physical properties of Hep-20 are listed in supplemental Table S1. Hep-20 was diluted in milli-Q water to obtain a stock solution of 8 mg/ml.

2.3 Fungicidal activity of Hep-20 in VFS
A vaginal fluid simulant mimicking human vaginal secretions was used to evaluate whether the activity of Hep-20 was maintained in such condition. The VFS was prepared as described by Owen and Katz and the solution pH adjusted to 4.5 using HCl, with the following composition: NaCl, 3.5 g/l; KOH, 1.4 g/l; Ca(OH)₂, 0.22 g/l; bovine serum albumin, 0.018 g/l; lactic acid, 2 g/l; acetic acid, 1 g/l; glycerol, 0.16 g/l; urea, 0.4 g/l; and glucose, 5 g/l (20).

The solution was then filtered through a 0.22 μm filter (Merck Millipore, Rome, Italy) and stored at -20°C prior to use. The fungicidal activity of Hep-20 against BPY44 was evaluated in a microdilution assay performed in VFS 4-fold diluted in sterile deionised water, to mimic average fluid dilution induced by vaginal suppository (21).

Briefly, *C. glabrata* BPY44 fluconazole resistant isolate was grown in Sabouraud medium to exponential phase at 30°C and suspended in deionised-water at a concentration of 1×10⁷ cells/ml. Ten microliters of fungal suspension were incubated in the presence of Hep-20 (concentrations ranging from 25 to 100 μM) in 100 μl of VFS with and without ethylenediaminetetraacetic acid (EDTA) a chelator of divalent cations (1 mM final concentration).
Following a 90 minute incubation, samples were diluted ten-fold in water and 200 µl were plated onto Sabouraud plates and incubated for 24 hours to determine CFU number. The fungicidal effect was defined as a reduction in the number of viable yeast cells $\geq 3 \log_{10}$ CFU/ml, in comparison to the untreated control (11, 13).

2.4 HVF extraction

HVF was collected from three healthy donors (D1-D3), who ranged in age from 26 to 42 years and had regular menstrual cycles of approximately 28 days. To account for protein content variability over the menstrual cycle, HVF was collected twice from two of the donors, before and after ovulation. The human vaginal fluid was collected as described by Valore and co-workers (22), with minor modifications: tampons (O.B. Pro ComfortTM MINI) were inserted in the vagina for 8 to 10 hours and then transferred into centrifuge tubes. Thirty ml of 10 mM SPB, pH 5.0, were added to each tampon and incubated under agitation at 37°C. The fluid retained in tampons following a 4 hour incubation was squeezed out by centrifuging at 3800 × g to remove epithelial cells and tampon debris. In order to reverse the extreme dilution due to the extraction procedure, the fluid was centrifuged through a 3 KDa filter spin unit (Merck, Millipore) for 3-4 hours up to a final volume of 1 ml. The effective protein concentration of HVF was then measured by Lowry assay, versus a bovine serum albumin (BSA) standard solution (100 mg/l BSA stock solution) (23). HVF was then diluted appropriately (final protein concentration 2g/l) (24) and stored at -20°C until further use. The study protocol was approved by the Local Ethics Committee and all the volunteers gave their informed written consent.

2.5 Fungicidal activity of Hep-20 in HVF

The fungicidal activity of Hep-20 against BPY44 was evaluated by killing assay performed in HVF, quantified as described above and diluted to a protein content of 2 g/l (24). C. glabrata BPY44 was grown in Sabouraud medium to exponential phase at 30°C and suspended in deionised water at a concentration of $1 \times 10^5$ cells/ml. Fungal suspensions were incubated in the presence of Hep-20 (ranging from 3.12 to 50 µM) in a 4-fold diluted HVF in sterile deionised water with and without...
1.5 mM EDTA (final volume of 100 µl). Following incubation for 15 minutes to 24 hours at 37°C, each mixture was diluted ten-fold in water and 200 µl plated onto Sabouraud plates and incubated for 24 hours to determine CFU number. The fungicidal effect was defined as a reduction in the number of viable yeast cells \( \geq 3 \) Log\(_{10}\) CFU/ml, in comparison to the untreated control (11).

2.6 Evaluation of Hep-20 degradation in HVF

Hep-20 (5 µg) was incubated in four-fold diluted HVF from D1-D2 for 90 minutes and 24 hours, respectively, at 37°C under agitation in the presence and absence of EDTA. Four-fold diluted HVF with and without 1.5 mM EDTA were included in the analysis as controls. Hep-20 (5 µg) incubated alone was used as negative control of degradation. Following incubation, samples and controls were boiled for 10 minutes in SDS-loading buffer (50 mM TrisCl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and separated on 17.5% Tricine/SDS-PAGE gels (25). Molecular weight marker (7-175 kDa) and samples were processed for 2 hours in stacking gel (4 % polyacrylamide, Sigma-Aldrich) at 10 mA and 4 hours at 30 mA in separating gel. Following electrophoresis gels were stained with EZ blue reagent (Sigma Aldrich). Bands were visualised with the Image Master apparatus (VDS, Ge Health Care, Milan, Italy). To evaluate the contribution of HVF proteases to Hep-20 degradation, experiments were performed in parallel in pre-treated vaginal fluid to 100°C for 5 minutes to inactivate proteases (26).

2.7 Peripheral blood mononuclear cell (PBMC) isolation and A549 culturing

Heparinized venous blood obtained from 6 healthy volunteers was diluted in phosphate-buffered saline (PBS, Euroclone, Milan, Italy) containing 10% (vol/vol) sodium citrate (Sigma-Aldrich) and layered on a standard density gradient (Lympholyte®-H, Cedarlane®, Euroclone). Following centrifugation at 160 × g for 20 minutes at room temperature, supernatant was removed, without disturbing the lymphocyte/monocyte layer at the interface, in order to eliminate platelets. The gradient was further centrifuged at 800 × g for 20 minutes, and peripheral blood mononuclear cells (PBMC) were collected from the interface. Cells were washed three times with PBS containing 0.1% (wt/vol) BSA (Sigma-Aldrich) and 10% sodium citrate (Sigma-Aldrich), and suspended in...
RPMI 1640 (Euroclone) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (FCS, Euroclone) at a final cell concentration of $1 \times 10^6$ cells/ml. Human non-small cell lung adenocarcinoma A549 cells (ATCC® Number CCL-185™, LGC Standards, Sesto San Giovanni, Italy) were cultured in tissue culture flasks in D-MEM (Euroclone) supplemented with 2 mM L-glutamine and 10% heat-inactivated FCS. Cell cultures were maintained in a 5% CO$_2$ atmosphere at 37°C. When cells grew to confluence, they were treated with Trypsin (Sigma-Aldrich) and split in new flasks.

2.8 PI staining of PBMCs and A549 cell line incubated with Hep-20

Cell viability was analysed by flow cytometry, by evaluating PI incorporation in PBMCs and A549 cells after a 24 hour exposure to Hep-20. Isolated PBMCs were seeded in a 96-well microtiter plate (1 × 10$^5$ cells/well) and Hep-20 added to wells at a final concentration of 6.25-100 μM. Cells incubated with complete medium alone served as negative control, while PBMCs incubated with cycloheximide (Sigma-Aldrich) (2 mg/ml final concentration) were used as positive control. Plates were incubated in a 5% CO$_2$ atmosphere at 37°C for 24 hours. Cells were then transferred into 12 × 75 mm polystyrene tubes and washed in D-PBS (Euroclone) and 5 μl of a 50 mg/l solution of PI (Sigma-Aldrich) were added to each tube. Following an incubation at room temperature for 4 minutes in the dark, 15,000 events were acquired ungated in a flow cytometer (FACSort, BD Biosciences, Mountain View, CA, USA). CellQuest software (BD Biosciences) was used for computer assisted analysis. In order to evaluate PI incorporation by A549, cells were detached with trypsin from culture flask, counted and suspended in D-MEM supplemented with 10% FCS and 2 mM L-glutamine (5 × 10$^4$ cells/ml final concentration). Two hundred microliters from the suspension were seeded in a 96-well microtiter plate (1 × 10$^4$ cells/well), and cells cultured in a 5% CO$_2$ atmosphere at 37°C for 24 hours to allow adhesion. Fifty microliters of complete medium containing Hep-20 or cycloheximide at desired concentrations were then added to each well. Following a further 24 hour incubation, cells were harvested by trypsinization, washed and exposed to PI and analysed as described for PBMCs. Experiments were repeated three times. The percentage...
of cytotoxicity was calculated by subtracting spontaneous PBMCs/A549 cell-death, measured in cultures in the absence of Hep-20 or cycloheximide, according to the following formula: 

\[
\text{cytotoxicity} = \left( \frac{\text{PI positive cells}_{\text{SAMPLE}} - \text{PI positive cells}_{\text{SPONTANEOUS}}}{\text{PI positive cells}_{\text{SPONTANEOUS}}} \right) \times 100.
\]

2.9 XTT reduction assay on PBMCs and A549 cell line incubated with Hep-20

The effect of Hep-20 on cell viability was also assessed using a colorimetric assay based on the reduction of the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide tetrazolium salt (XTT) into a water soluble orange formazan product by mitochondrial dehydrogenases of viable cells. The XTT-PMS solution was prepared according to Scudiero and colleagues (27). In order to improve cellular reduction of XTT, N-methylphenazonium methyl sulphate (PMS), an electron coupling agent, was used. Briefly, XTT (Sigma-Aldrich) was prepared at 1 mg/ml in pre-warmed (37°C) D-PBS and PMS at 5 mM in D-PBS (1.53 mg/ml) (Sigma-Aldrich). A 0.025 mM PMS-XTT solution was prepared mixing 5 ml of fresh XTT (1 mg/ml) and 25 μl of 5 mM PMS immediately before use, and sterilized by filtration (0.22 μm, Millipore).

PBMCs and A549 cells were suspended in complete medium and seeded in a 96-well microtiter plate as described above. Cells were then incubated with Hep-20 at desired concentrations (6.25-100 μM final concentrations), complete medium alone as negative control and triton-X 100 (Sigma-Aldrich, Milan, Italy) (0.5% v/v final concentration) as positive control. After a 24 hour incubation in presence of 5% CO₂, 50 μl of the XTT-PMS solution were added to each well. Following a further incubation (3 hours and 1 hour for PBMCs and A549, respectively) the optical density (OD₄₅₀ nm) was measured using a microplate reader (Model 550, BIO-RAD, Milan, Italy), in order to quantify the formazan production, which gave an indirect measure of cell viability. Experiments were performed in duplicate and repeated three times.

2.10 Statistical analysis
Statistical analysis was performed using Instat software (GraphPad, USA). One-way ANOVA was used to evaluate the activity of Hep-20 in VFS, HVF and for cytotoxicity studies. A P value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Fungicidal activity of Hep-20 in VFS

In order to evaluate whether the activity of Hep-20 was maintained in a biologic fluid simulant, killing assays on C. glabrata BPY44 were performed in a 4-fold diluted artificial fluid (VFS) with Hep-20 concentrations ranging from 25 to 100 μM. Hep-20 did not produce any fungicidal effect (at least 3 log reduction in CFU number) at any of the concentrations tested (data not shown), but a significant reduction in BPY44 CFU number was observed only with the highest peptide concentration (Fig. 1). To evaluate whether the reduced activity of the peptide was due to the presence of inhibitory cations, experiments were repeated in 4-fold diluted VFS supplemented with the chelating agent EDTA. Preliminary experiments performed using different EDTA concentrations (0.75 - 4 mM) indicated that 1 mM EDTA potentiated the peptide activity. As shown in Figure 1, the addition of 1 mM EDTA to VFS significantly enhanced the antifungal activity of Hep-20 against C. glabrata at concentration of 100 μM following 90 minute incubation period (One-way ANOVA; P<0.0001), demonstrating its fungicidal potential.

3.2 Antifungal activity of Hep-20 in HVF

Killing experiments were performed in 4-fold diluted human vaginal fluid (HVF) collected from 3 healthy donors (D1-D3). The total protein content in HVF was quantified by Lowry assay and the fluid was diluted to obtain a protein concentration of 2 g/l. This value was chosen to represent an average protein content of HVF, which was reported to vary between 0.018 g/l to 3.75 g/l (24).
When the peptide was co-incubated for 90 minutes at 37°C with *C. glabrata* in the presence of HVF from D1, no antifungal activity of Hep-20 was observed up to a concentration of 50 µM. Indeed, as shown in Figure 2A, no significant difference was observed between untreated control and treated samples for all the peptide concentrations tested (12.5 to 50 µM). Notably, this result was not confirmed when fluid from donor 2 was used in the killing assay: in this case a highly significant reduction in *C. glabrata* CFU number could be evidenced in HVF (One-way ANOVA, P<0.0001) (Fig. 2B). Fluid from donor 3 gave an intermediate result with a significant decrease in HVF fungal burden (One-way ANOVA, P=0.0003) (Fig. 2C), following 90 minutes of incubation at 37°C. In the attempt to restore the peptide activity, which could have been hampered by ions concentration, experiments were performed in parallel using human vaginal fluid supplemented with EDTA.

Preliminary experiments performed on 4-fold diluted HVF and different EDTA concentrations indicated that 1.5 mM EDTA potentiated the Hep-20 activity in HVF producing a fungicidal effect with 50 µM Hep-20 (data not shown).

Co-incubation of Hep-20 with HVF from the three donors in the presence of 1.5 mM EDTA restored fungicidal activity, following 90 minutes of incubation at 37°C, with a minimal fungicidal concentration (MFC) of 25 µM (Fig. 2A, 2C) and 12.5 µM (Fig. 2B). Although non fungicidal, even the lowest peptide concentration used (12.5 µM) caused a significant reduction in BPY44 CFU number in vaginal fluid collected from D1 and D3 (Fig. 2 A, 2 C, P<0.001).

Experiments were repeated using vaginal fluid from donor 1 and 2 collected before and after ovulation. For each of the two donors the results obtained in HVF collected before ovulation did not significantly differ from those obtained with HVF collected post ovulation (data not shown).

Time killing assay of 12.5 µM and 25 µM Hep-20 versus BPY44 performed in HVF from donor 1 confirmed that the highest peptide concentration exerts a fungicidal effect following 90 minute incubation in the presence of 1.5 mM EDTA (Fig. 3). Notably a statistically significant reduction in fungal cell number (P<0.05) was observed starting at 15 minutes of incubation with 25 µM...
Hep-20 and at 30 minutes with 12.5 µM Hep-20 (Fig. 3). When co-incubation was prolonged to 24 hours, Hep-20 produced a fungicidal effect also at a lower concentration (12.5 µM) (Fig. 3).

3.3 Evaluation of Hep-20 degradation in HVF

The stability of Hep-20 was evaluated by co-incubating the peptide in the presence of HVF collected from two healthy donors (D1-D2). The peptide was incubated in the presence of 4-fold diluted HVF for 90 minutes and 24 hours at 37°C. In parallel, the same experiment was performed on HVF pre-heated at 100°C for 5 minutes to inactivate proteases in the fluid. Samples including HVF+Hep-20 with and without 1.5 mM EDTA, HVF+EDTA, Hep-20 alone (5 µg), Hep-20 alone incubated for 90 minutes and 24 hours, were separated in 17.5 % tricine SDS-PAGE gel. As shown in Figure 4A and 4B, no band corresponding to Hep-20 molecular weight (lane 1 and 2) could be observed in 4-fold diluted HVF (lanes 7 and 8, Fig. 4A and 4B). Co-incubation for 90 minutes and 24 hour at 37°C of the peptide with HVF from D1 (lane 3 and 4, Fig. 4A and 4B) resulted in a partial degradation of Hep-20, in comparison with the peptide alone (lane 1 and 2, Fig. 4A and 4B). Pre-heating of HVF resulted in a complete lack of peptide degradation (lane 5 and 6, Fig. 4A and 4B). The same results were obtained with HVF from donor 2, with a similar extent of peptide degradation (data not shown).

3.4 Propidium iodide staining

The potential cytotoxic activity of Hep-20 was assessed by PI staining of PBMCs and A549. To this aim, PBMCs and A549 cell suspensions were incubated for 24 hours in the presence of Hep-20 at concentrations ranging from 6.25 to 100 µM and in the presence of 2 mg/ml cycloheximide as positive (death) control. PBMCs and A549 cellular suspensions were co-incubated with PI for 4 minutes and then processed by flow cytometry. Hep-20 produced a negligible cytotoxic effect at all the concentrations tested on PBMCs and A549, with more than 90% viable cells post-treatment.
3.5 XTT reduction assay

The cytotoxicity of Hep-20 on PBMCs and on a human derived epithelial cell line (A549) was also assessed by XTT assay. PBMCs and A549 cell suspensions were incubated for 24 hours in the presence of different concentrations of Hep-20 (6.25 to 100 μM) and in the presence of 0.5 % Triton-X as positive (death) control. Following this incubation, PBMCs and A549 cellular suspensions were co-incubated with XTT for 3 hours and 1 hour respectively at 37°C, and O.D.λ_{450} nm values provided information on cell metabolic activity. Notably, all the Hep-20 concentrations tested (6.25 to 100 μM) did not show any cytotoxic effect on PBMCs and A549 cells (Fig. 5A and B). Instead, a stimulatory effect could be observed at the highest peptide concentration tested, which resulted statistically significant in the case of A549 (Fig. 5A, One-way ANOVA, P=0.0033).

4. DISCUSSION

Candida glabrata vaginitis is difficult to treat due to an intrinsic reduced susceptibility to azoles, in particular to fluconazole, which is widely used for the topical treatment of this mycosis (1, 28). For this reason, there is an urgent need to individuate novel strategies for the treatment of these mycoses. In this regard, the therapeutic potential of natural anti-infective agents, such as AMPs, has generated much interest due to the potential therapeutic approaches that these molecules offer (5).

The present study was focused on human Hep-20, which was demonstrated to exert antimicrobial activity against clinically relevant Gram positive and Gram negative bacterial species (13).

We have recently shown that Hep-20 produced a fungicidal effect on C. glabrata clinical isolates with different levels of fluconazole susceptibility (11). This finding was particularly interesting in view of the scarce susceptibility this species presents to AMPs as well as to azoles (3, 7-10). Furthermore the enhanced fungicidal activity of Hep-20 observed under acidic conditions suggested that this peptide could be used as potential therapeutic agent of C. glabrata related...
infections in body districts characterized by low pH, such as vaginal district (11). No data are currently available with regard to the mode of action of Hep-20 on C. glabrata. Indirect information can be extrapolated from a recent study based on the interaction between Hep-20 and E. coli (29). This study demonstrates a greater tendency of the peptide to destabilize E. coli membrane under acidic conditions than at neutral pH, through formation of pores as evidence by the release of β–galactosidase from Hep-20 treated cells at pH 5.0 and formation of blebs (29).

To verify the applicability of the peptide in the vaginal environment, the antifungal activity of Hep-20 was measured in an artificial vaginal fluid simulating human vaginal secretions (VFS). Considering that the average volume of vaginal fluid ranges from 0.5 to 0.75 ml (21), in our experiments VFS was diluted 4-fold to mimic fluid dilution by a vaginal suppository (20, 21).

In this experimental condition, the Hep-20 fungicidal effect previously observed in SPB (11) was not confirmed for any of the concentrations tested (25 to 100 µM). This finding suggested that Hep-20 activity could be inhibited by the presence in the medium of VFS components such as BSA and mono and divalent cations such as Ca²⁺, K⁺ and Na⁺ (20). Indeed, the antimicrobial activity of several AMPs has been shown to be markedly reduced in biological fluids due to the presence of proteins, other components or physiological concentration of salts, such as K⁺, Na⁺, Mg²⁺ and Ca²⁺ (14-17).

Among the different mechanisms proposed to explain the loss of antimicrobial activity of AMPs in biological fluids, it has been hypothesized that the presence of divalent cations such as calcium and magnesium, mediates an electrostatic interaction with the negatively charged membrane of microorganisms. This creates a barrier preventing the interaction between AMPs and microbial plasma membrane. For this reason, in the attempt to restore the activity of the peptide in VFS, experiments were repeated in the presence of 1 mM EDTA. This synthetic chelating agent forms strong complexes with cations and it is widely used as a stabilizer in food and also in pharmaceutical vaginal applications (30, 31). In vitro studies have demonstrated that prolonged exposure of ectocervical tissue to EDTA can produce a toxic effect at concentrations higher than 2.5
mM (31). Furthermore, EDTA has been demonstrated to inhibit hyphal development in *C. albicans* and to exert antifungal activity against *S. cerevisiae* at concentrations of 10 mM and 17 mM, respectively (30, 32). In a study performed on human saliva by Wei and Bobek, the addition of EDTA enhanced the antifungal activity of different peptides such as MUC7, histatin 5 and magainin against *C. albicans*, in comparison with saliva alone (14).

In our experiments, the addition of EDTA to the VFS resulted in a significant enhancement of Hep-20 fungicidal activity against *C. glabrata* BPY44, following 90 minutes of incubation at 30°C. This finding reinforced the view that divalent cations inhibit the activity of Hep-20: the presence of EDTA most likely enhanced Hep-20 activity by removing cations that protect cell membrane from interaction with the peptide or by sequestering ions which are required by fungal intracellular enzymes (14, 30).

This finding was particularly interesting in view of a potential application of this peptide in the topical treatment of fungal vaginitis. To further pursue this possibility the fungicidal activity of Hep-20 was evaluated in HVF collected from 3 healthy donors (D1-D3) and diluted to a final protein concentration of 2 g/l to consider HVF biological variability (24).

Killing experiments in HVF indicated that a biological variability occurred among vaginal fluid collected from three different donors, since for two of them a significant reduction in the number of viable fungal cells was induced by Hep-20 in the fluid alone following 90 minutes of incubation at 37°C. However, in the case of D1, no antifungal activity was observed in HVF in the absence of EDTA. This finding indicates that vaginal fluid composition may differentially affect the peptide activity. Indeed, it is recognised that HVF contains several AMPs such as calcoprotectin, lysozyme, SLPI, HBD1-2, HNP1-3 and LL-37 which could act synergistically with Hep-20, thus overcoming the natural inhibitory effect caused by ions or other fluids components (22, 26, 33).

Notably, when 1.5 mM EDTA was added to the HVF, Hep-20 exerted a complete fungicidal effect versus *C. glabrata* BPY44 following 90 minutes of incubation, and it was maintained after 24 hour incubation. Interestingly, Hep-20 MFC that produced a fungicidal effect was 25 µM for all donors,
a quarter of the peptide concentration that significantly reduced fungal cell number in VFS. In addition experiments performed using HVF collected before and after ovulation gave similar results for each of the donors considered, suggesting that variation of HVF composition during the menstrual cycle does not significantly influence Hep-20 activity. Overall, the results obtained on Hep-20 fungicidal activity in VFS, and, most importantly, in HVF, reinforced the view that this peptide is a promising candidate for the topical treatments of *C. glabrata* vaginal infections.

In order to assess the potential of Hep-20 as a therapeutic agent against *C. glabrata* vaginal infections, we investigated whether the peptide retains its activity in physiological conditions. The results obtained in this study demonstrated that Hep-20 maintains its antifungal activity in the presence of EDTA at pH, salt concentration and protein content similar to those found in vivo (VFS). Moreover, this finding was also confirmed when the peptide activity was evaluated in HVF. At present, no data are available with regard to the stability of the peptide in HVF. An indirect evidence of Hep-20 stability was obtained in this study from co-incubation experiments of the peptide with HVF at 37°C at two different time points, 90 minutes and 24 hours. SDS-PAGE gels indicated that when the peptide was co-incubated with HVF supplemented or not with 1.5 mM EDTA, Hep-20 was partially degraded at the two time points considered. However, when the peptide was co-incubated with pre-heated HVF, Hep-20 gave a band similar in intensity to the untreated peptide, suggesting that the partial degradation observed was mainly due to fluid trypsin-like proteases. Indeed, a proteomic analysis on cervico-vaginal fluids identified several proteases, such as serine (e.g., kallikreins) and cysteine (e.g., cathepsins) ones, which could degrade Hep-20 (26, 33). Further experiments will be required to understand the exact mechanism leading to this partial peptide degradation following 90 minutes and 24 hours of incubation in HFV. Nevertheless, killing experiments performed in HVF clearly demonstrated that, even if partially degraded, the peptide is still able to exert fungicidal activity against *C. glabrata* at both 90 minutes and within 24 hours. However, the partial degradation of the peptide may be overcome by synthesizing hepcidin-
derived peptides containing D-amino acids and/or by incorporating these peptide analogues in nanoparticles, thus enhancing the bioavailability of Hep-20 in HVF.

Even if Hep-20 is a human derived peptide, therapeutically active concentrations can be higher than physiological ones. Therefore, it is mandatory to investigate the potential cytotoxic effects exerted by this peptide on human cells. Previous data reports a lack of hemolytic effect on human erythrocytes (11).

In this study we expanded upon the Hep-20 cytotoxicity experiments, by evaluating the effect of different peptide concentrations on human peripheral blood mononuclear cells (PBMCs) and a human derived epithelial cell line (A549) by PI staining and XTT reduction assay. The results obtained showed that all Hep-20 concentrations tested (6.25 to 100 µM) produced negligible or no cytotoxic effect on PBMCs and A549 cells, as demonstrated by PI staining and XTT assay. These results are in agreement with a study by Park and co-workers in which Hep-20 have been tested for cytotoxicity on erythroleukemia type K562 human cells at concentrations up to 30 µM (about 3000 fold higher than that found in urine), with no significant effect on human cells (88% viable cells post treatment) (19).

It is worth underlying that Hep-20 produced a significant increase in A549 cell metabolism at the highest concentration tested (100 µM). This result is not surprising, since it is reported that some antimicrobial peptides, such as hBD-2, are able to induced A549 proliferation in a dose dependent manner (35). In addition, the role of AMPs in wound healing is supported by data on the activity of cathelicidin LL-37 and hBD-2 and hBD-3 (36-38). These peptides are highly expressed in epidermal keratinocytes in response to injury or infection of the skin (36-38). It has also been shown that treatment with exogenous hBD-3 leads to an enhanced re-epithelialization of wounds in animal models (38). Hep-20 shares a high structural homology with defensins, and it could be hypothesized to exert a similar stimulatory effect on A549 cells (39).

In conclusion, despite the fact that further studies are still needed to characterize the peptide mode of action and to optimize Hep-20 fungicidal activity, our results indicate that this peptide or
its derivatives should be further studied as novel therapeutic agents for the control of vaginal C. glabrata infections.

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References


Legends to Figures

Figure 1: Killing assay of *C. glabrata* BPY44 in 4-fold diluted VFS with and without 1 mM EDTA, in the presence of 100 µM Hep-20. Fungal cells incubated in the absence of Hep-20 serve as viable control (CTRL). Data are expressed as mean ± standard deviation of three independent experiments. ***P<0.0001.

Figure 2: Fungicidal activity of Hep-20 against *C. glabrata* BPY44 in 4-fold diluted HVF from (A) Donor 1 (D1), (B) Donor 2 (D2) and (C) Donor 3 (D3). Fungal cells incubated in the absence of Hep-20 serve as viable control (CTRL). Data are expressed as mean ± standard deviation of at least three independent experiments. The arrows indicate fungicidal effect. NS, not significant. ***P<0.0001, **P<0.01 and *P<0.05.

Figure 3: Time kill curves of Hep-20 (12.5 µM and 25 µM) against *C. glabrata* BPY44 in 4-fold diluted HVF from D1 with and without 1.5 mM EDTA. Fungal cells incubated in the absence of Hep-20 served as viable control (CTRL). Data are expressed as mean ± standard deviation of three independent experiments. The arrows indicate fungicidal effect. *P<0.05; **P<0.01.

Figure 4: SDS-PAGE gels of the peptide alone and co-incubated in the presence of HVF from donor D1 following (A) 90 minutes in HVF at 37°C and (B) 24 hour incubation in HVF at 37°C. Lanes correspond to: **M** (molecular marker 7-175 kDa), 1) Hep-20 (5 µg), 2) Hep-20 (5 µg) + 1.5 mM EDTA, 3) HVF + Hep-20, 4) HVF+1.5 mM EDTA + Hep-20, 5) HVF (pre-heated at 100°C for 5 minutes) + Hep-20, 6) HVF (pre-heated at 100°C for 5 minutes) + Hep-20+1.5 mM EDTA, 7) HVF+ 1.5 mM EDTA, 8) HVF (pre-heated at 100°C for 5 minutes) + 1.5 mM EDTA.
Figure 5: OD values (λ 450 nm) of formazan salt production by (A) A549 cells and (B) PBMCs incubated with concentrations of Hep-20 ranging from 6.25 to 100 µM. Triton-X 100 (0.5% v/v) served as positive control. The vitality percentage, shown for each condition, was calculated considering negative control as 100% viability. Data are expressed as mean of three separate experiments ± standard error of mean. *** P=0.0033.