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## Candidacidal activity of synthetic peptides based on the antimicrobial domain of the neutrophil-derived protein, CAP37

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### Abstract

The primary bactericidal domain of CAP37, a cationic antimicrobial protein with potent activity against Gram-negative organisms was previously shown to reside between amino acids 20 through 44 (NQGRHFCCGALIHARFVMTAASCFQ) of the native protein. In this study, we explored the efficacy of four synthetic CAP37 peptide analogs, based on this sequence, against various *Candida* species including fluconazole-sensitive and -resistant isolates of *C. albicans*. Three of the peptides demonstrated strong antifungal activity for *C. albicans*, including fluconazole-resistant isolates of *C. albicans* and were active against *C. guilliermondii*, *C. tropicalis*, *C. pseudotropicalis*, *C. parapsilosis*, and *C. dubliniensis*. The peptides were ineffective against *C. glabrata*, *C. krusei*, and *Saccharomyces cerevisiae*. For *C. albicans* isolates, the peptides had relatively greater activity against blastoconidia than hyphal forms, although strong antifungal activity was observed with pseudohyphal forms of the various *Candida* species tested. Kinetic studies demonstrated fungicidal rather than fungistatic activity. These findings indicate that synthetic peptides based on the antimicrobial domain of CAP37 also have activity against eukaryotic organisms suggesting a broader range of activity than originally demonstrated and show for the first time their potent fungicidal activity.

### Keywords

CAP37; antibiotic peptides; *in vitro* fungicidal activity; *Candida albicans*; innate immunity

### INTRODUCTION

CAP37 (cationic antimicrobial protein of  $M_r$  37 kDa), also termed heparin binding protein [1] and azurocidin [2], was originally identified as a component of the oxygen-independent killing mechanism of the human neutrophil (PMN) and demonstrated strong bactericidal activity for Gram-negative organisms, including *Salmonella typhimurium*, *Escherichia coli*,

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and *Pseudomonas aeruginosa* [3,4,5]. Distinct from its effect on bacteria the native CAP37 protein has potent regulatory effects on host cells. It is an effective regulator of cells of the mononuclear phagocytic system such as monocytes [6], microglia [7], and macrophages [8]. It also regulates corneal epithelial [9], endothelial [10,11], and smooth muscle cell functions [12].

Structure function analysis of CAP37 enabled us to delineate its antibacterial activity to a domain consisting of 25 amino acids corresponding to residues 20 through 44 of the native molecule [13]. A synthetic peptide (CAP37<sub>20-44</sub>) based on this sequence mimicked the antimicrobial activity of the native molecule [13] and demonstrated an extended range of activity to encompass two Gram-positive organisms, *Staphylococcus aureus* and *Enterococcus faecalis*. *In vivo* experiments demonstrated the efficacy of CAP37<sub>20-44</sub> in attenuating the lethal effects of *E. coli* lipopolysaccharide (LPS) in a rat model of endotoxic shock [14]. The synthetic peptide bound and neutralized the toxic effects of LPS resulting in the attenuation of many of the circulatory and metabolic dysfunctions and lethality associated with sepsis [14]. The combination of sequence, charge, amphipathicity and hydrophobicity appear to be unique to CAP37<sub>20-44</sub>, since two synthetic peptides, based on cathepsin G and neutrophil elastase, share almost 40% sequence identity in this region and were significantly less effective at killing bacteria and binding LPS [13,14].

In this study we extended our findings on the antimicrobial repertoire of the synthetic peptide CAP37<sub>20-44</sub> to determine whether it possessed antifungal activity as a means to evaluate its effects against eukaryotes. We further explored the importance of the cysteine residues by synthesizing two new analogs of CAP37<sub>20-44</sub>. One analog had the cysteine at position 26 substituted with a serine residue (CAP37<sub>20-44Ser26</sub>), and the other analog had the cysteine at position 42 replaced with a serine residue (CAP37<sub>20-44Ser42</sub>). The activities of these three analogs were compared to a fourth peptide in which both cysteine residues were replaced by serine residues (CAP37<sub>20-44ser26/42</sub>). The latter peptide had previously been shown to be ineffective against Gram-negative organisms [13]. The various CAP37 peptide analogs were tested for activity against fluconazole-sensitive and -resistant clinical isolates of *Candida albicans*, a number of other *Candida* species and two isolates of *Saccharomyces cerevisiae* to determine its range of activity. Additionally, the efficacy of the peptide on hyphal forms of *C. albicans* and pseudohyphal forms of *C. pseudotropicalis* and *C. tropicalis* was determined along with a series of time-kill studies to establish whether the activity was fungicidal or fungistatic.

## MATERIALS AND METHODS

### Synthesis of peptides based on CAP37

Peptides were synthesized by tert-butyloxycarbonyl (Boc)/benzyl solid-phase synthesis on an Applied Biosystems peptide synthesizer (model 433A, Foster City, CA) as previously described [6]. PAM-resins were used and activation of Boc-amino acids was accomplished using HOBt/DCC. The side-chain-protected, Boc-L-amino acids included: Boc-Arg (mesitylsulfonyl); Boc-Cys(4-methoxybenzyl) for Cys; Boc-His(dinitrophenyl), Boc-His (benzyloxycarbonyl) or Boc-His(benzoxymethyl) for His; Boc-Ser(benzyl); and Boc-Thr (benzyl). The couplings were performed in either dimethylformamide or N-methylpyrrolidone/dimethyl sulfoxide as solvents. The peptides were cleaved off the resin and deprotected in liquid HF/anisole/dimethyl sulfide (10:1:0.5) for 1 h at 0°C, precipitated with cold diethyl ether, extracted into 50% aqueous acetic acid, and lyophilized. The crude peptides were dissolved in formic acid/TFA/trifluoroethanol/water (20:1:10:70, v/v) at approximately 15 mg/ml and purified by preparative reversed-phase (RP) HPLC on a Zorbax SB-C18 silica column (22×250 mm, 5 µm particle size, 300 Å pore size; Agilent, Palo Alto, CA) equilibrated in 0.1% aqueous TFA and eluted using a linear gradient of acetonitrile in

0.08% aqueous TFA at 25°C. Purity of the peptides was ascertained by analytical RP-HPLC using a C18 silica SUPER-ODS column (2.1×100 mm, 2 µm particle size, 300 Å pore size; TosoHaas, Montgomeryville, PA), eluted using a gradient of acetonitrile in 0.1% aqueous TFA and monitored at 214 nm. It confirmed our observations made during preparative RP-HPLC, namely that CAP37<sub>20-44</sub> and its derivatives aggregate in highly acidic solutions and do not chromatograph as sharp, distinct peaks on C18 packings; instead they elute as broad peaks in the 40% and 50% acetonitrile concentration range. Counter-ion alternatives to TFA, such as heptafluorobutyric acid or formic acid, and/or alternative elution solvents to acetonitrile, such as 2-propanol or 1-butanol, did not improve the elution profiles of these peptides, and neither did column elution at higher temperatures (up to 65°C). The HPLC fractions were screened by MALDI-TOF mass spectrometry (MS) and those containing the desired product, as determined by its presence as the major base peak in the mass spectra corresponding to the desired product, were pooled. The purified peptides were obtained lyophilized in the form of their TFA salts. The purity and integrity of the peptides was confirmed by MALDI-TOF MS and electrospray ionization MS, and by N-terminal Edman sequencing, using described protocols [15].

Peptides were synthesized predicated on previous findings that a synthetic peptide, CAP37<sub>20-44</sub>, based on the native amino acid sequence of CAP37 consisting of residues 20 through 44 (NQGRHFCCGALIHARFVMTAASCFQ) had potent bactericidal activity for a number of Gram-negative organisms [13]. CAP37<sub>20-44</sub> contains two cysteine residues in positions 26 and 42 which form a disulfide bridge in the native CAP37 molecule [16]. Previously we showed that simultaneous replacement of both cysteines with serine residues (CAP37<sub>20-44ser26/42</sub>) abrogated antibacterial activity [13]. This peptide is routinely incorporated in our in vitro studies to serve as an inactive or control peptide. The synthesis of CAP37<sub>20-44</sub> is technically challenging due to problems related to solubility and aggregation in the purification stage. We thus queried whether a peptide with replacement of a single cysteine would retain its antimicrobial activity yet overcome the purification problems we had encountered. Therefore, in this study, we synthesized two new analogs containing singly substituted cysteine residues. The analog in which the cysteine at position 26 was replaced by a serine is indicated as CAP37<sub>20-44ser26</sub> and the analog in which the cysteine at position 42 was replaced by a serine is indicated as CAP37<sub>20-44ser42</sub>. The replacement of either one or both cysteine residues is considered a very conservative replacement [17]. The peptides used in this study were obtained with cysteine residues in their reduced form. We confirmed, using mass spectrometry that under the conditions used for the assay (pH 5.5, see below) no significant oxidation of the peptides occurred in the stock solutions prior to their use or upon several weeks of storage at -20°C.

### Fungal isolates and culture conditions

Isolates used in this study included *C. albicans* (3153A), three fluconazole-sensitive isolates of *C. albicans* and three fluconazole-resistant isolates of *C. albicans* from patients with vulvovaginitis; and a blood isolate of *C. albicans* obtained from a patient with candidemia. Isolates were stored frozen at -70°C in 10% glycerol and streaked onto Sabouraud Dextrose Agar (SAB, Sigma, St. Louis, MO) plates and maintained on plates at 4°C, with subculturing approximately every 10 days for the duration of the studies. Fluconazole resistance as determined by standard in vitro susceptibility assays was defined as the MIC of >64 µg/ml. Three clinical isolates of *C. glabrata*; two clinical isolates of *C. dubliniensis*; single isolates each of *C. krusei*, *C. guilliermondii*, *C. parapsilosis*, *C. pseudotropicalis*, and *C. tropicalis*; and two isolates of *Saccharomyces cerevisiae* were also used in this study and were maintained similarly on SAB plates at 4°C. For use in assays, a single yeast colony was cultured at 33°C in 1% phytone peptone broth (Becton Dickinson, Sparks, MD) with 0.1% D-glucose (Sigma) to stationary phase (~ 18 h or overnight). Blastospores were collected and enumerated by trypan blue dye exclusion.

## Induction of hyphae

To induce hyphal formation, a single colony of each *C. albicans* isolate was cultured as above to stationary phase. An aliquot (500  $\mu$ l) of the stationary phase culture was subcultured in 5 ml of RPMI-1640 (Cellgro Mediatech Inc, Herndon, VA) with 10% fetal calf serum (Invitrogen, Grand Island, NY) for 120 min at 37°C with rotation. Cultures were monitored and assessed for hyphal formation using phase microscopy. The *C. albicans* isolates under these growth conditions were predominantly mycelium but with some still in transition from germ tube to hyphae [18]. *C. pseudotropicalis* and *C. tropicalis* demonstrated pseudohyphal morphology under the growth conditions described. Samples were normalized by optical density reading at 600 nm for addition to the antifungal assays as described below. Enumeration of samples was confirmed by plating 100  $\mu$ l of the culture at the start of the assay (T=0).

## Standard antifungal colony forming unit (CFU) assay

A standard CFU assay [2,4,13] was performed to assess the antifungal activity of the peptides incorporating the following modifications. Stock solutions (1mg/ml) of all peptides were made in sterile endotoxin-free water for irrigation (Baxter, Deerfield, IL). Subsequent dilutions were all made in tryptone saline pH 5.5 [3]. An aliquot (100  $\mu$ l) of a stationary phase fungal culture was subcultured further in 5 ml of 1% phytonone peptone broth with 0.1% glucose and incubated in a shaking water bath (80 oscillations per min, Precision, Winchester, VA) for 90 min at 33°C to yield a logarithmic culture. Cell cultures under these conditions were found to consist predominantly of blastoconidia as determined by phase contrast microscopy. The optical density was determined and the culture adjusted to 500 blastoconidia/100  $\mu$ l in tryptone saline pH 5.5 [3]. To 100  $\mu$ l of the organism suspension in a 96 well sterile polystyrene microtiter plate (Becton Dickinson, Franklin Lakes, NJ) was added 100  $\mu$ l of the peptide at varying concentrations for an initial dose response and further at final concentrations of 400  $\mu$ g/ml and 200  $\mu$ g/ml. In addition, two controls, T=0 and T=240, were set up consisting of 100  $\mu$ l of organism suspension and 100  $\mu$ l of tryptone saline. 100  $\mu$ l from the T=0 control was plated immediately and served to confirm the starting numbers of the organisms used in each assay. The microtiter plate was incubated at 37°C for 4 h and 100  $\mu$ l of the contents from each well was plated on SAB plates and incubated at 37°C overnight. The CFU were counted and the antifungal activity was expressed as percent inhibition and calculated according to the following equation:  $[(T_{240}\text{control CFU} - \text{test CFU})/T_{240}\text{control CFU}] \times 100 = \% \text{ inhibition}$ .  $T_{240}\text{control CFU}$  was an indication of the number of colonies present following 240 min of incubation in tryptone saline alone in the absence of peptide. Test CFU was determined by counting the number of colonies present after incubation in tryptone saline containing the peptide. Each experimental point was performed in triplicate. Assays were conducted on blastoconidia unless otherwise indicated.

## Time-kill assays

Kinetic fungicidal assays were performed using *C. albicans* (3153A), *C. pseudotropicalis*, *C. glabrata*, and *S. cerevisiae*. CAP37<sub>20-44</sub> and control peptide, CAP37<sub>20-44ser26/42</sub> at concentrations ranging from 400 to 100  $\mu$ g/ml were incubated with the four isolates listed above for 2, 5, 15, 30, 60 and 240 min and cell death measured using the standard antifungal CFU assay.

## Statistical analysis

Data are presented as mean  $\pm$  standard error from three or four independent experiments. Statistical analysis of the data was performed employing one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test, Tukey's Multiple Comparison Test, or Bonferroni Multiple Comparison Test using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).  $P < 0.05$  was considered significant.

## RESULTS

### Standardization of *in vitro* CFU assay

*C. albicans* (3153A) was used to initiate and standardize the *in vitro* antifungal assay. We explored a range of peptide concentrations (750, 500, 400, 200 and 100 µg/ml), contact time between peptide and *Candida* (0.5, 1, 2, and 4 h), optimal growth conditions to achieve log phase cultures of *Candida* (temperature and time, 25°C for 5 h and 33°C for 90 min), and numbers of blastoconidia per well (200, 400, 500, 600, 800, and 1000 CFU) in the standard 4 h assay. Peptides are considered inactive at inhibition of <40%. Results in Figure 1, depict a dose response analysis using 500 CFU *C. albicans* and a 4 h incubation time with peptide. There was good distinction between the activities of the active (CAP37<sub>20-44</sub>) and the control peptide (CAP37<sub>20-44ser26/42</sub>) at 400, 200, and 100 µg/ml. We selected peptide concentrations of 400 (150 µM) and 200 µg/ml (75 µM) for our standard assay. We selected 4 h as the routine time of exposure of *Candida* to the peptides based on findings that the inactive peptide required 120 to 240 min to reach maximum activity. These data are fully discussed in the detailed time-kill studies described later in the results section and are not duplicated here. Data (not shown) indicated that there was no significant difference between *Candida* that had been cultured for 5 h at 25°C or for 90 min at 33°C. The 90 minute incubation was more convenient technically and was therefore employed routinely. Analysis of the number of blastoconidia per well indicated that 500 CFU was optimal. The standard assay thus consisted of using 500 CFU per well of *Candida* that had been grown to log phase at 33°C for 90 minutes and incubated for 4 h at 37°C with peptides at concentrations of 400 and 200 µg/ml. Any variations from these standard conditions are indicated in the relevant figure legends.

### Antifungal activity of CAP37 peptides on various *Candida* species

Using the standard CFU assay, we analyzed all four peptides for their antifungal activity on a number of different species of *Candida* and two isolates of *S. cerevisiae*. Differential activity of the CAP37 peptides was observed depending on the species of *Candida* tested (Figure 2). CAP37<sub>20-44</sub>, CAP37<sub>20-44ser26</sub>, and CAP37<sub>2044ser42</sub> were significantly more active than CAP37<sub>20-44ser26/42</sub> on all isolates of *Candida* tested (P<0.001). CAP37<sub>20-44</sub>, CAP37<sub>20-44ser26</sub> and CAP37<sub>2044ser42</sub> were highly effective against *C. guilliermondii* and *C. parapsilosis* at concentrations as low as 100 µg/ml (37.5 µM) (Figure 2) compared to control peptide (P<0.001). *C. pseudotropicalis*, was also extremely sensitive to peptides CAP37<sub>20-44</sub>, CAP37<sub>20-44ser26</sub>, and CAP37<sub>20-44ser42</sub> compared to the control peptide (P<0.001). Surprisingly, some activity, although significantly less than with the other three peptides, was also obtained against *C. pseudotropicalis* with the control peptide CAP37<sub>20-44ser26/42</sub>, considered as ineffective against Gram-negative organisms. CAP37<sub>20-44ser26/42</sub> lacked activity (% inhibition ≤ 40%) against all other *Candida* species and *S. cerevisiae* isolates tested. *C. tropicalis*, *C. albicans*, and *C. dubliniensis* were also sensitive to CAP37<sub>20-44</sub>, CAP37<sub>20-44ser26</sub> and CAP37<sub>20-44ser42</sub> (P<0.001 compared to CAP37<sub>20-44ser26/42</sub>). Similar to *C. guilliermondii* and *C. pseudotropicalis*, there was no significant difference between the activity of the three active peptides for *C. tropicalis* and *C. albicans*. However, CAP37<sub>20-44</sub> was significantly less active than CAP37<sub>20-44ser26</sub> and CAP37<sub>20-44ser42</sub> against *C. parapsilosis* (P<0.001) and the two isolates of *C. dubliniensis* (P<0.01 and 0.001). Activity against the three *C. glabrata* isolates, the single isolate of *C. krusei*, and the two isolates of *S. cerevisiae*, was considered minimal to negative when compared to the activity of the peptides against all the other isolates tested.

### Antifungal activity of CAP37 peptides on fluconazole-sensitive and -resistant isolates of *C. albicans*

Having established the activity of the CAP37 peptides against the panel of *Candida* species, we next evaluated their antifungal activity against three fluconazole-sensitive and three

fluconazole-resistant clinical mucosal isolates of *C. albicans*. We found that the peptides CAP37<sub>20-44</sub>, CAP37<sub>20-44ser26</sub> and CAP37<sub>20-44ser42</sub> were strongly active against the fluconazole-sensitive isolates (Figure 3a–c) at a concentration of 400 µg/ml (~150 µM) when compared to the activity demonstrated by the control peptide CAP37<sub>20-44Ser26/42</sub> ( $P < 0.001$ ). There was no statistical difference ( $P > 0.05$ ), as determined by ANOVA followed by Tukey's Multiple Comparison Test, between the activities of the three peptides (CAP37<sub>20-44</sub>, CAP37<sub>20-44ser26</sub> and CAP37<sub>20-44ser42</sub>) at 400 µg/ml for any given fluconazole-sensitive isolate. Furthermore, there was no statistical difference ( $P > 0.05$ ) in the activity of each peptide for the three different fluconazole-sensitive isolates. Activity was negligible with CAP37<sub>20-44</sub>, CAP37<sub>20-44ser26</sub> and CAP37<sub>20-44ser42</sub> at 200 µg/ml.

The antifungal activity of peptides CAP37<sub>20-44</sub>, CAP37<sub>20-44Ser26</sub>, CAP37<sub>20-44Ser42</sub>, and CAP37<sub>20-44ser26/42</sub> against the three fluconazole-resistant isolates of *C. albicans* are shown in Figure 3d–f. Peptides CAP37<sub>20-44</sub>, CAP37<sub>20-44Ser26</sub>, and CAP37<sub>20-44Ser42</sub> were highly active against the three fluconazole-resistant isolates compared with peptide CAP37<sub>20-44ser26/42</sub> ( $P < 0.05$  with isolate in Figure 3d and  $P < 0.001$  with isolates in Figures 3e and 3f). Significant activity ( $P < 0.05$ ) was also obtained with the lower concentration (200 µg/ml or 75 µM) of peptides, CAP37<sub>20-44</sub>, CAP37<sub>20-44Ser26</sub>, and CAP37<sub>20-44Ser42</sub>, with the fluconazole-resistant isolate in Figure 3d. Although, CAP37<sub>20-44ser26/42</sub> peptide at 400 µg/ml was considerably less active than the other peptides with this isolate, it demonstrated moderate activity exhibiting approximately 58% inhibition. There were no significant differences in antifungal activity between each of the three active peptides for any of the fluconazole-resistant isolates.

#### Antifungal activity of CAP37 peptides on hyphal forms of *C. albicans*

To determine whether CAP37 peptides could inhibit hyphal growth, we evaluated the activity against the same fluconazole-sensitive and -resistant *C. albicans* isolates following conditions for hyphal development (Figure 4a–f). Of the various CAP37 peptides tested, CAP37<sub>20-44ser42</sub> was significantly inhibitory against hyphal forms of all 6 isolates tested, when compared with the control peptide, CAP37<sub>20-44ser26/42</sub> ( $P < 0.05$  with isolate in Figure 4a, and  $P < 0.01$  with all other isolates). CAP37<sub>20-44</sub> was significantly inhibitory against the hyphal form of isolates in figures 4b and 4f ( $P < 0.01$ ) and figure 4e ( $P < 0.05$ ). On the other hand, CAP37<sub>20-44ser26</sub> was not effective against any of the hyphal forms when compared to activity with the control peptide.

#### Antifungal activity of CAP37 peptides on pseudohyphal forms of *C. pseudotropicalis* and *C. tropicalis*

In contrast to the limited activity displayed by the CAP37 peptides against the hyphal forms of *C. albicans*, we observed potent activity against the pseudohyphal form of *C. pseudotropicalis* with peptides CAP37<sub>20-44</sub>, CAP37<sub>20-44Ser42</sub>, CAP37<sub>20-44Ser26</sub>, and CAP37<sub>20-44ser26/42</sub> at 400 and 200 µg/ml (Figure 5a) and *C. tropicalis* (Figure 5b) with all four peptides at 400 µg/ml. Limited activity was observed against *C. tropicalis* using 200 µg/ml (Figure 5b).

#### Time-kill studies

Kinetic assays were performed with three different *Candida* species and one isolate of *S. cerevisiae* using CAP37<sub>20-44</sub> at three concentrations (400, 200 and 100 µg/ml) and CAP37<sub>20-44ser26/42</sub> at two concentrations (400 and 200 µg/ml). Antifungal activity of CAP37<sub>20-44</sub> at 400 µg/ml for *C. albicans* (Figure 6a) showed a dramatic increase between 2 and 15 min and reached a plateau of >85 % between 30 and 60 minutes, reaching maximum antifungal activity thereafter. The lower concentration of CAP37<sub>20-44</sub>, showed lesser activity at the earlier time points, but displayed significant activity after 240 minutes of incubation. Activity of CAP37<sub>20-44</sub> was significantly quicker and greater than CAP37<sub>20-44ser26/42</sub> at the

comparable concentration. The rapid antifungal activity of CAP37<sub>20-44</sub> was more pronounced on *C. pseudotropicalis*. Significant levels of activity were observed as early as two minutes (Figure 6b). In contrast, and as expected from data depicted in figure 2, the activity of both peptides was negligible against *C. glabrata* (Figure 6c) and *S. cerevisiae* (Figure 6d) at all time points tested.

## DISCUSSION

In this study, we demonstrate for the first time that synthetic peptides based on the CAP37 molecule have strong activity against eukaryotic organisms. We had previously shown that the 25 amino acid peptide based on the native sequence had activity against a range of prokaryotic organisms [13]. Here we describe the synthesis of two new CAP37-derived peptide analogs in which the cysteine residues at positions 26 or 42 were replaced by serine residues. Our findings demonstrate significant activity against several *Candida* species by the peptide based on the native sequence and the two new analogs. The replacement of both cysteines significantly abrogated activity suggesting that a single cysteine residue is needed for the antifungal activity of the peptides. The concentration of CAP37 peptides required to achieve antifungal activity is approximately twice that required for activity against the Gram-negative species *S. typhimurium*, *E. coli*, and *P. aeruginosa* [13], although we used the same number of CFU in both the antifungal and antibacterial assays. This range in activity is not uncommon amongst cationic antimicrobial peptides, which tend to have specificity and greater potency towards certain bacterial species, fungi, parasites and/or viruses. Fungicidal activity on sensitive isolates was relatively rapid requiring between 5 to 240 minutes contact time depending on the organisms. We had previously proposed that the bactericidal mechanism of CAP37 and its antimicrobial peptides on Gram-negative organisms was due to the specific binding to the lipid A component of the endotoxin molecule [13,14]. The putative target molecule on yeast cells that acts as the receptor for the CAP37 peptides is presently unknown. It is possible that charge, hydrophobicity, and other non-specific factors alone may contribute to this binding. Alternatively, it is possible that there may be a specific binding site on yeast for CAP37. It has recently been demonstrated that salivary histatin-5 kills *C. albicans* through a process that requires binding to Ssa1/2 proteins on the cell surface [19]. Clearly the structure function analysis of this complex peptide and its mode of action on bacteria and fungi require further exploration.

Our data showed that the peptides exhibited potent activity against *C. guilliermondii* and *C. parapsilosis* at lower concentrations (37.5 µM) compared to that for the other species. *C. parapsilosis* is most often isolated from critically ill patients in intensive care units who typically have indwelling catheters and devices while undergoing treatment [20,21]. Almost 100% of the starting inocula of *C. pseudotropicalis* and *C. tropicalis* were killed with peptide concentrations of 150 µM. *C. tropicalis* is being increasingly isolated from blood cultures of patients with leukemias, other neoplasias, and those in intensive care units [22]. Of the peptides, CAP37<sub>20-44Ser42</sub> and CAP37<sub>20-44Ser26</sub> were more effective against *C. dubliniensis* than peptide CAP37<sub>20-44</sub> which was based on the native sequence. *C. dubliniensis* is a recently identified species that is seldom found in healthy persons but tends to be found as the causative agent of oropharyngeal infections mainly in HIV-infected individuals [23]. The peptides were ineffective against *C. glabrata*, *C. krusei* and *S. cerevisiae*. *C. glabrata* and *C. krusei* are isolated with increasing frequency from blood stream infections and *C. krusei* in particular appears to be associated with hematological malignancies [24,25,26].

Noteworthy were our findings that CAP37<sub>20-44</sub>, CAP37<sub>20-44Ser26</sub>, and CAP37<sub>20-44Ser42</sub> peptides were very effective against the fluconazole resistant mucosal isolates. Overall, fluconazole-resistant and – sensitive species were inhibited nearly equivalent by the peptides. These findings are particularly pertinent to the recent emergence of fluconazole-resistant

isolates of *C. albicans* and other *Candida* species that will require treatment with new therapeutics with novel or alternative mechanisms of action [23,27]. In addition to the important finding that CAP37 peptides had activity against fluconazole-resistant mucosal isolates of *C. albicans*, the peptides showed impressive activity against pseudohyphae. On the other hand, activity of the peptides against the hyphal forms was modest compared to the respective blastoconidia forms. It is unclear whether the differences between pseudohyphae and hyphae are due to the morphological forms or the species. Obviously, further studies will be needed to answer this question. A critical finding was that the activity of the peptides on susceptible isolates was fungicidal rather fungistatic. This was shown both by kinetic studies as well as vital staining (data not shown). This is an important consideration if CAP37 peptides are to be pursued as potential therapeutics, since a number of current antifungal drugs are fungistatic and not fungicidal.

Besides CAP37, several cationic antimicrobial proteins including the defensins, hCAP18/LL37, bactericidal permeability increasing protein (BPI), and lactoferrin [5,28,29] are active against *C. albicans* [30,31]. Another well known candidacidal molecule is salivary histatin-5 [32,33] which is active on *C. albicans* blastospores at a concentration of 15  $\mu$ M [34]. In addition, synthetic peptides based on naturally occurring proteins such as the first cationic domain of the amino-terminus of human lactoferrin, Lfpep, a lactoferricin H-derived synthetic peptide, and Kaliocin-1 based on residues 153-183 of the human lactoferrin molecule, also show strong fungicidal activity [35,36] in the range of 50 to 150  $\mu$ M levels [35]. Synthetic histatin analogs based on the C-terminal fungicidal domain of histatin-5 are effective against *C. albicans* [37], *C. krusei*, fluconazole-resistant isolates of *C. glabrata* and *Aspergillus fumigatus* [38]. Similar to these antifungal peptides, optimal conditions for in vitro killing by CAP37 peptides required the use of media containing low salt concentrations [36] and pH of 5.5 [39].

The use of natural cationic peptides as novel therapeutics in the treatment of infections is gaining enthusiasm in the scientific and biotechnology communities. The major drawback to conventional antibiotics is the rapidity with which microorganisms can gain multiple resistance patterns. Although the exact mechanism as to how these cationic antimicrobial peptides, including the CAP37 peptides kill microorganisms is not entirely known, it is generally believed that membrane interaction and disruption occurs. Histatin-5 has been shown to cause small membrane defects, whereas LL-37 causes massive disruption of *C. albicans* cell membranes [40]. Although the mode of action of the CAP37 peptides has yet to be determined it appears to be different from the mechanism of action of azole-based drugs, since it kills fluconazole-resistant and -sensitive isolates equally well.

CAP37 has been traditionally considered a PMN-derived protein since it is constitutively expressed in the granules of these cells. However, more recently we have demonstrated the presence of an inducible form of CAP37 in endothelial cells lining the vasculature, smooth muscle cells, corneal epithelial cells, and squamous epithelial cells of the skin, [9,10,12,41,42]. Others have also demonstrated a wide repertoire of epithelial antimicrobial peptides in the skin [43,44,45,46], mucosal surfaces lining the gastrointestinal tract [47,48], oral surfaces [49,50], respiratory tract [51,52,53], and genitourinary tract [48,54]. These antimicrobial proteins are ideally located to serve as the first line of defense against invading pathogens. To date no cytotoxicity for mammalian cells, by CAP37 peptides, has been demonstrated. Whether expression of CAP37 occurs in mucosal linings of the host in response to *Candida* infections or whether the induction of CAP37 is compromised in mucosal and epithelial surfaces in patients with recurrent candidiasis, suggesting a physiological role for CAP37 in the protection of the host against fungal infections, is currently unknown. The study reported here describes for the first time that CAP37 peptides and analogs based on the native sequence of CAP37 possess potent fungicidal activity and suggest a more broad-spectrum antimicrobial activity



for the molecule than originally proposed. Its precise role in host defense against *Candida* infections will require investigation in appropriate animal models of Candidiasis.

## Acknowledgments

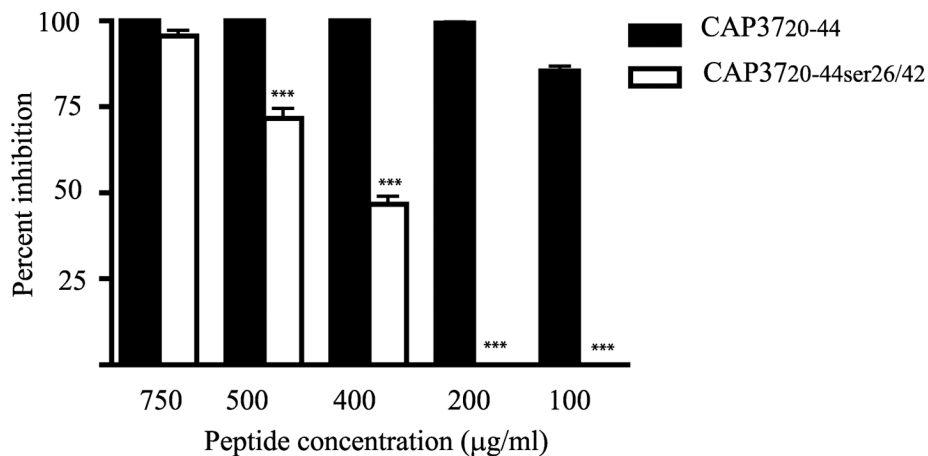
This study was supported in part by Department of Pathology funds to (HAP), and Public Health Service grants AI28018, EY015534 to (HAP), and DE12178 and AI32556 to (PLF) from the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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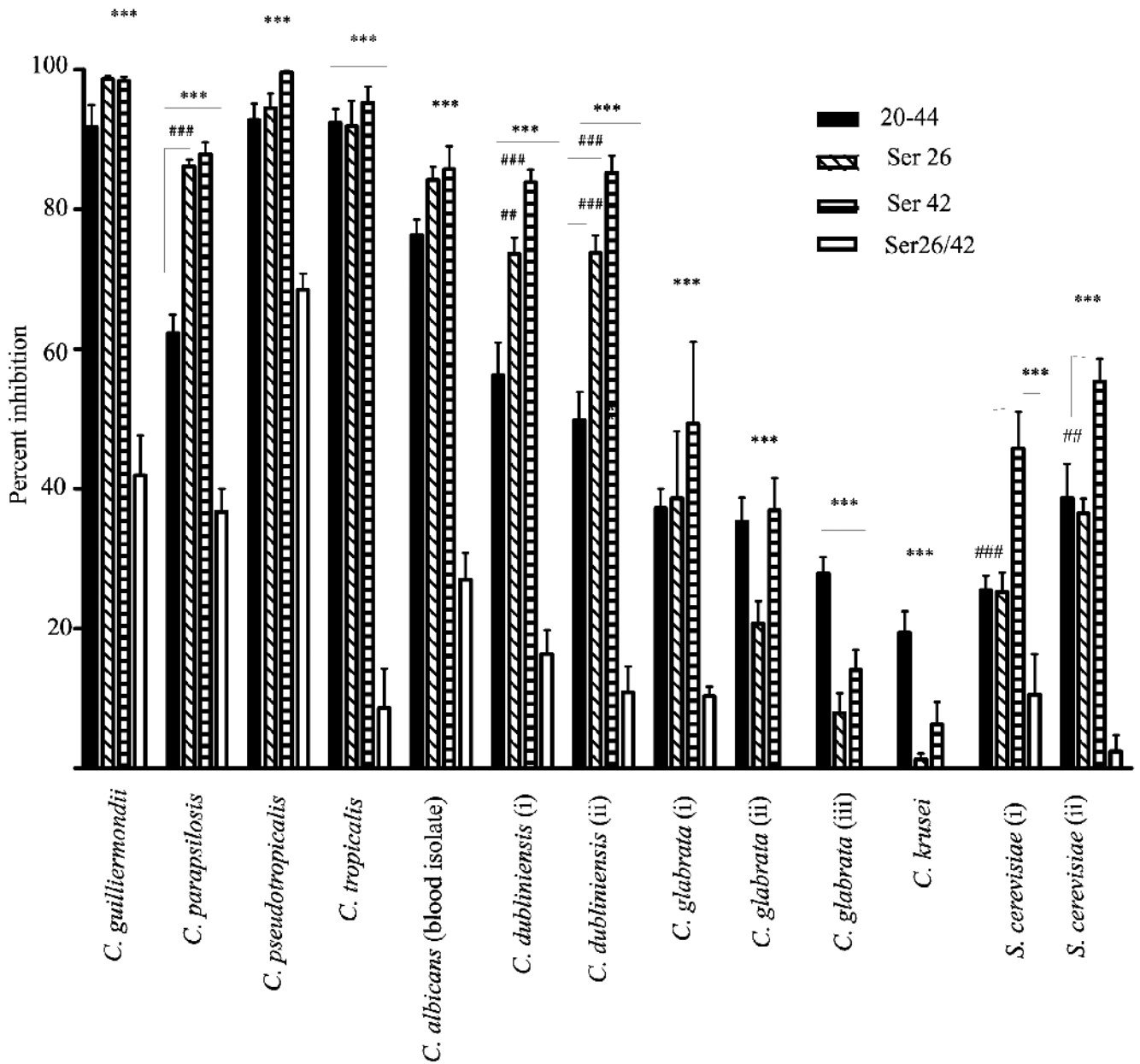
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**FIG 1. Standardization of peptide concentration for antifungal assays**

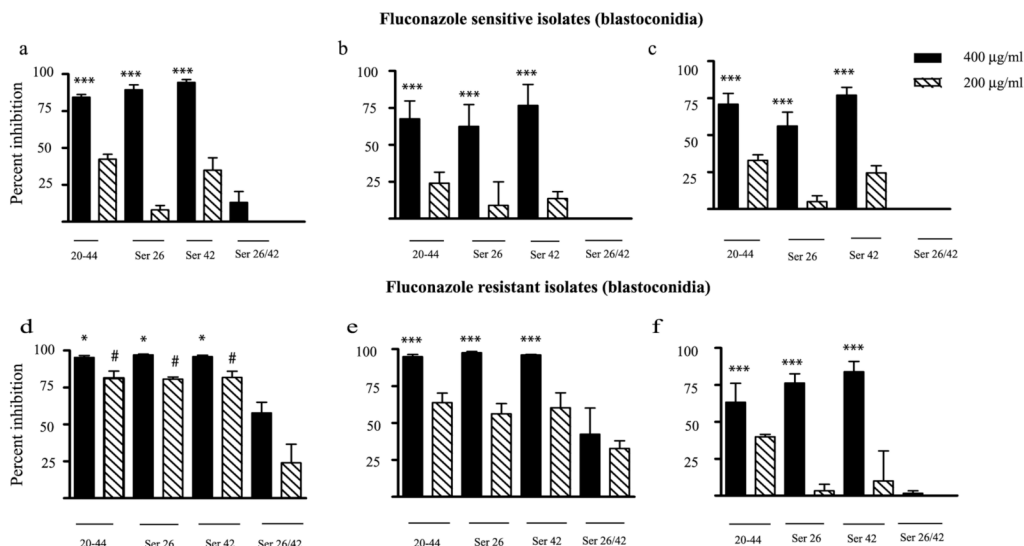
The antifungal activity of CAP37<sub>20-44</sub> and CAP37<sub>20-44ser26/42</sub> at concentrations ranging from 750 to 100 µg/ml was evaluated using *C. albicans* (3153A). Values are expressed as ± standard error of the mean from three independent experiments performed in triplicate. \*\*\* $P < 0.001$  compared to activity of CAP37<sub>20-44ser26/42</sub> at the same concentration using ANOVA followed by Bonferroni's multiple comparison test.



**FIG 2. Activity of CAP37 peptides against various *Candida* species**

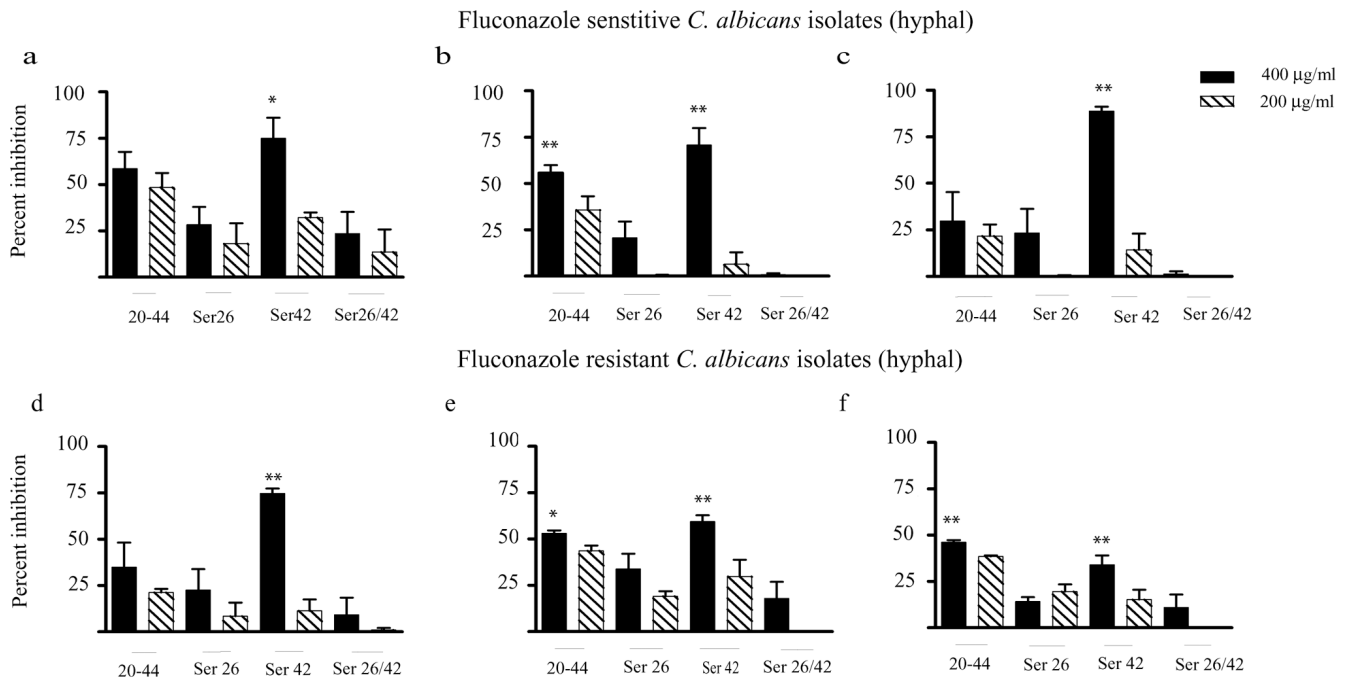
The antifungal activity of CAP37 peptides, CAP37<sub>20-44</sub> (20-44), CAP37<sub>20-44Ser26</sub> (Ser 26), CAP37<sub>20-44Ser42</sub> (Ser 42), and CAP37<sub>20-44Ser26/42</sub> (Ser 26/42) was evaluated using a panel of *Candida* species that included *C. guilliermondii*, *C. parapsilosis*, *C. pseudotropicalis*, *C. tropicalis*, a blood isolate of *C. albicans*, two isolates of *C. dubliniensis*, three isolates of *C. glabrata*, one isolate of *C. krusei* and two isolates of *S. cerevisiae*. Data reported here are for a single concentration of the four different peptides. The peptide concentration for *C. guilliermondii* and *C. parapsilosis* was 100 µg/ml (37.5 µM) whereas data for all other isolates were obtained using peptide concentrations of 400 µg/ml (150 µM). Values are expressed as ± standard error of the mean from three to four independent experiments performed in triplicate. Statistical analysis of data was performed using ANOVA followed by Tukey's Multiple Comparison Test., \*\*\*P<0.001 represent significance values between control peptide and other

peptides for an individual isolate. ##P<0.01 and ###P<0.001 represent significance values between CAP37<sub>20-44</sub> and peptides CAP37<sub>20-44ser26</sub> and CAP37<sub>20-44ser42</sub> for an individual isolate.



**FIG 3. Activity of CAP37 peptides against fluconazole-sensitive and -resistant isolates of *C. albicans* blastoconidia**

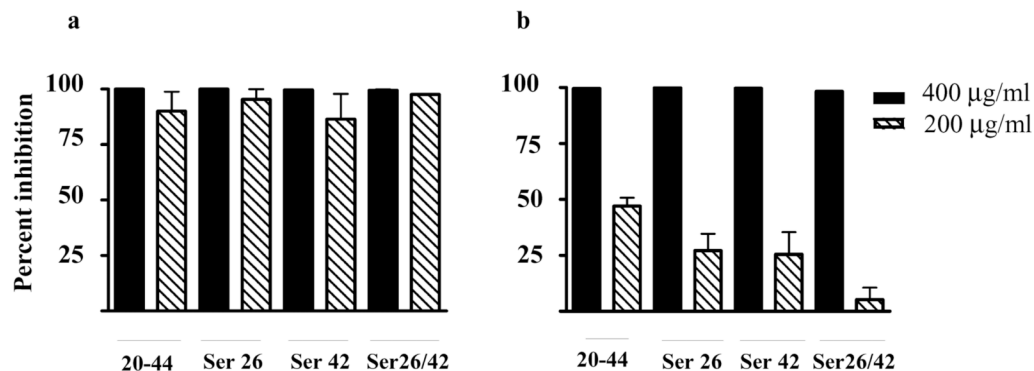
Activity of the four CAP37 peptides, CAP37<sub>20-44</sub> (20-44), CAP37<sub>20-44Ser26</sub> (Ser 26), CAP37<sub>20-44Ser42</sub> (Ser 42), and CAP37<sub>20-44Ser26/42</sub> (Ser 26/42) at 400 µg/ml (solid bars) and 200 µg/ml (diagonal striped bars) was evaluated against blastoconidia from three clinical isolates of *Candida albicans* known to be fluconazole-sensitive (a–c) and three isolates known to be fluconazole-resistant (d–e). The percent inhibition is determined as detailed in the Materials and Methods section. Values are expressed as ± standard error of the mean from three independent experiments performed in triplicate. \**P* < 0.05 and \*\*\**P* < 0.001 compared to activity with control peptide, CAP37<sub>20-44Ser26/42</sub> at 400 µg/ml and #*P* < 0.05 compared to activity with control peptide at 200 µg/ml using ANOVA followed by Dunnett’s Multiple Comparison Test.



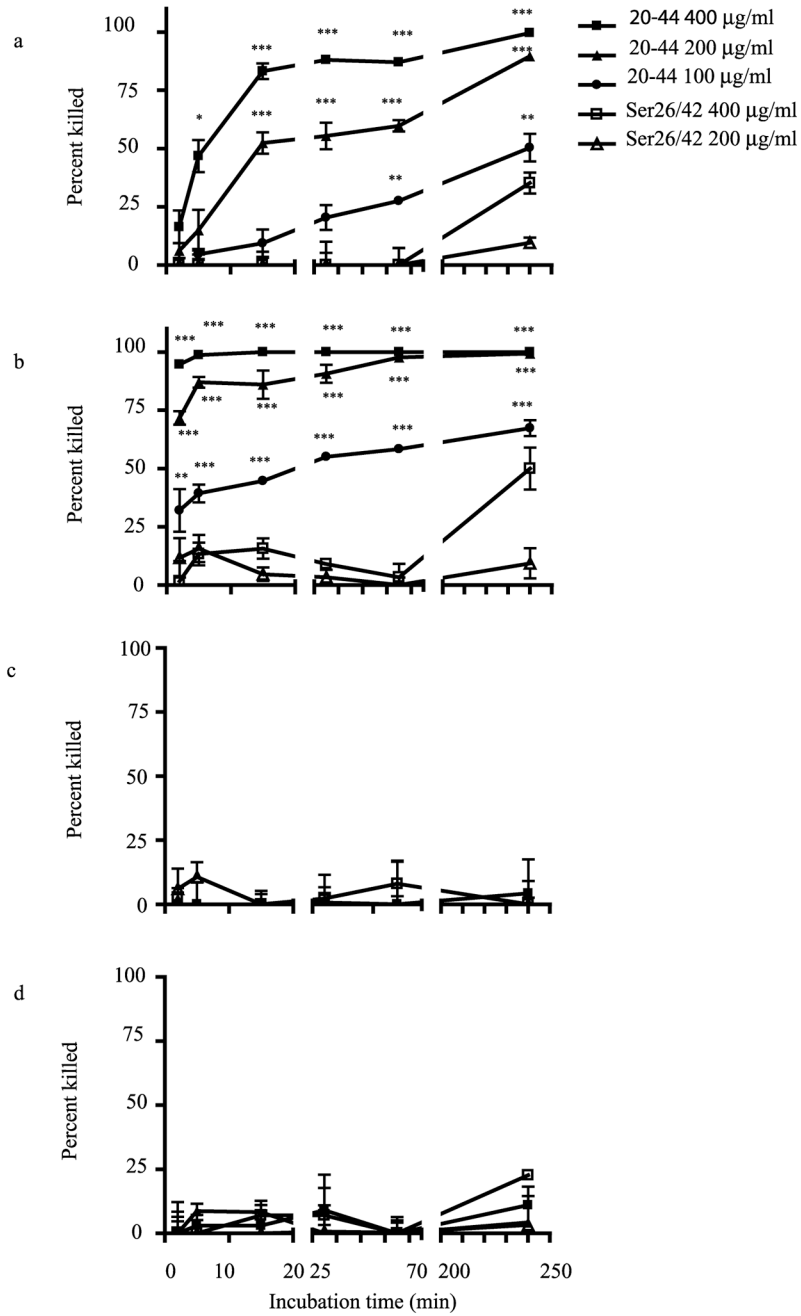
#### FIG 4. Activity of CAP37 peptides on *C. albicans* hyphae

Activity of CAP37 peptides CAP37<sub>20-44</sub> (20-44), CAP37<sub>20-44Ser26</sub> (Ser26), CAP37<sub>20-44Ser42</sub> (Ser42), and CAP37<sub>20-44Ser26/42</sub> (Ser26/42) at 400 µg/ml (solid bars) and 200 µg/ml (diagonal striped bars) was evaluated against hyphal forms of the clinical isolates of *Candida albicans*. The isolates used are identical to those in Figure 3 and were cultured so as to induce hyphal growth. Isolates depicted in Figure 4a–c are fluconazole-sensitive and those in Figure 4d–f are fluconazole-resistant. The percent inhibition was determined as detailed in the Materials and Methods section. Values are expressed as  $\pm$  standard error of the mean from three independent experiments performed in triplicate. \* $P < 0.05$  and \*\* $P < 0.001$  compared to activity with control peptide, CAP37<sub>20-44ser26/42</sub> at 400 µg/ml using ANOVA followed by Dunnett's Multiple Comparison Test.





**FIG 5. Activity of CAP37 peptides on pseudohyphal forms of *C. pseudotropicalis* and *C. tropicalis***  
 Activity of CAP37 peptides CAP37<sub>20-44</sub> (20-44), CAP37<sub>20-44ser26</sub> (Ser26), CAP37<sub>20-44ser42</sub> (Ser42) and CAP37<sub>20-44ser26/42</sub> at 400 (solid bars) and 200 µg/ml (diagonal striped bars) was evaluated using *C. pseudotropicalis* (a) and *C. tropicalis* (b) that were induced to form pseudohyphae as indicated in the methods section. The percent inhibition was determined as detailed in the methods section. Values are expressed as ± standard error of the mean from three independent experiments performed in triplicate. P>0.05 compared to activity with control peptide, CAP37<sub>20-44ser26/42</sub> at 400 and 200 µg/ml using ANOVA followed by Dunnett's Multiple Comparison Test.



**FIG 6. Time kill studies to determine fungicidal activity of CAP37 peptides**

*C. albicans* (3152A) (a), *C. pseudotropicalis* (b), *C. glabrata* (c, isolate iii in Figure 2), and *S. cerevisiae* (d, isolate ii in Figure 2) were selected for the kinetic studies. The fungal isolates were incubated with active peptide CAP37<sub>20-44</sub> (20-44) at concentrations of 400, 200 and 100 µg/ml, and CAP37<sub>20-44ser26/42</sub> (Ser26/42) at 400 and 200 µg/ml for 2, 5, 15, 30, 60, and 240 min. CFU were counted and the antifungal activity determined as described in the methods and materials section. Values are expressed as ± standard error of the mean from three or five independent experiments performed in triplicate. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared to activity with CAP37<sub>20-44ser26/42</sub> using ANOVA followed by Bonferroni's Multiple Comparison Test.