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Effect of Sequence and Structural Properties on 14-Helical β-Peptide Activity against *Candida albicans* Planktonic Cells and Biofilms

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ABSTRACT β-Peptides (β-amino acid oligomers) that mimic the amphiphilic, helical, and cationic properties of natural antimicrobial peptides have previously been shown to display antifungal activity against planktonic *Candida albicans* cells. β-Peptides offer several advantages over conventional peptides composed of α-amino acid residues, including conformational stability, resistance to proteases, and activity at physiological salt concentrations. We examined sequence–activity relationships toward both planktonic *C. albicans* cells and *C. albicans* biofilms, and the results suggest a toxicity mechanism involving membrane disruption. A strategy for fluorescently labeling a β-peptide without diminishing antifungal activity was devised; labeled β-peptides penetrated the cell membrane and accumulated in the cytoplasm of both planktonic and biofilm-associated cells. The labeled β-peptide was detected only in metabolically inactive cells, which suggests that β-peptide entry is correlated with cell death. The presence of a β-peptide at a concentration near the minimum inhibitory concentration completely prevented planktonic *C. albicans* cells from forming a biofilm, suggesting that β-peptides may be useful in preventing fungal colonization and biofilm formation.

Candida albicans is among the most commonly isolated fungal pathogens (*1*), causing cutaneous, mucosal, and systemic infections. Although several antifungal drugs are available for treating candidiasis, they act on relatively few distinct molecular targets, and resistance to these drugs is a frequent problem when treating systemic infections (*2*). The ability of *C. albicans* to grow as a biofilm on medical devices, such as catheters and dentures, further complicates treatment, since biofilms exhibit increased resistance to antifungal therapies (*3*). The resistance of pathogens such as *C. albicans* to current antifungal therapies, along with the increasing prevalence of opportunistic pathogens, has heightened the need for new therapeutics that provide safe and effective treatments for fungal infections.

One class of molecules that has potential for development of new antifungal agents is antimicrobial peptides. These peptides provide a natural defense against a broad spectrum of pathogens, including Gram-positive bacteria, Gram-negative bacteria, and fungi (*4*). Although they are diverse in terms of sequence and structure, antimicrobial peptides usually bear a net positive charge and are able to adopt a globally amphiphilic conformation, such as an α-helix in which hydrophobic and cationic side chains are segregated on different sides (*5*). The mechanisms of action for helix-forming antimicrobial peptides are not completely understood, but in most cases their biological effects are believed to involve membrane disruption of the target cells (*6, 7*). The proposed mechanism requires positively charged

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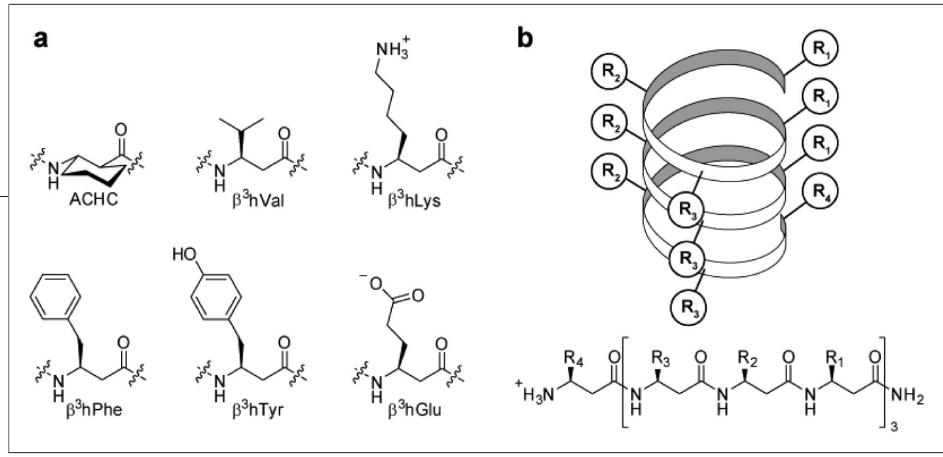

Figure 1. β-Peptide building blocks and secondary structure. a) β-Amino acid residues incorporated into the β-peptides listed in Table 1. Except for ACHC, all residues have side chains identical to their α-amino acid analogues. b) Three-dimensional representation of the β-peptide 14-helix.

peptides to associate with negatively charged microbial membranes, causing the peptides to adopt a globally amphiphilic helical conformation at the membrane–water interface. The ensuing membrane disruption can lead directly to cell lysis and death (*8, 9*), or membrane permeabilization may allow peptide molecules to reach intracellular targets (*10, 11*).

Development of resistance to antimicrobial peptides is thought to be more difficult than development of resistance to traditional antimicrobial agents (*6*). Traditional antimicrobial drugs, including amphotericin B and fluconazole, often act on specific biomolecular targets, and fungi can resist the action of such drugs through a variety of mechanisms, including mutations in the gene encoding the target or upregulation of drug efflux pumps (*12–14*). In contrast, antimicrobial peptides usually do not have a specific molecular target but instead are thought to kill *via* nonspecific interactions that compromise the cell membrane.

Natural antimicrobial peptides are susceptible to degradation by cellular proteases, and many have low conformational stability in aqueous solution (*15*), lack specificity for microbial targets over host cells (*7*), and exhibit little activity at physiological salt concentrations (*16*). Synthetic oligomers that adopt helical conformations have been designed to overcome these limitations of natural peptides (*17–21*), although studies of these oligomers' efficacy have focused mainly on antibacterial activity. For example, β-peptides (oligomers of β-amino acids) have proven to be effective mimics of α-helical antimicrobial peptides (*17–20*). The backbone of a β-amino acid contains an additional carbon atom relative to the α-amino acid backbone, which provides an additional site for side chain introduction and makes the β-peptides resistant to degradation by natural proteases (*22*). β-Peptides can adopt a variety of helical secondary structures, including the 14-helix, which contains 14-membered ring hydrogen bonds formed between the oxygen of the backbone C═O at position *i* and the hydrogen of the backbone H–N at position $i - 2$ (*23*). The cyclic constraint of *trans*-2-aminocyclohexanecarboxylic acid (ACHC) residues strongly stabilizes the β-peptide 14-helical conformation (*24–26*).

We recently reported that 14-helical β-peptides designed to be cationic and globally amphiphilic exhibit antifungal activity against planktonic *C. albicans* (*27*). Some of the β-peptides were selective for *C. albicans*, causing a low level of hemolysis at their minimum inhibitory concentrations (MICs) (*27*). The β-peptides were active at physiological ionic strength, and antifungal activity varied as a function of length and 14-helical propensity. In the current study, we demonstrate activity of β-peptides against *C. albicans* biofilms, and we elucidate structural features that regulate activity against planktonic and biofilm-associated *C. albicans*. Net charge and N-terminal capping affect β-peptide activity, but chirality does not. Using a fluorescently labeled β-peptide that maintains antifungal activity, we have observed that β-peptides enter *C. albicans* cells and that this entry correlates with loss of metabolic activity. Finally, we have shown that *C. albicans* biofilms exhibit diminished susceptibility to β-peptide-mediated mortality compared to planktonic cells but that β-peptides may be effective at preventing biofilm formation.

RESULTS AND DISCUSSION

β-Peptide Design. We synthesized β-peptides that are predicted to adopt the 14-helical conformation and studied how the sequences and structural properties of the β-peptides affect their antifungal activity. The β-amino acid residues we used are shown in Figure 1, panel a, and the β-peptide sequences and nomenclature are provided in Table 1. All single letter amino acid codes refer to β^3-homoamino acids that have the same side chain as the corresponding α-amino acids. Most β-peptides contain a repeating segment of three β-amino acid residues. Because the 14-helix has approximately three residues per turn, using a triplet repeat leads to alignment of identical side chains along the sides of the 14-helix (Figure 1, panel b). By varying the identities of the residues (*e.g.*, hydrophobic, cationic, anionic) in the repeating unit, we varied the prop-

TABLE 1. Sequences and MICs of β-peptides used in this study

β-Peptide	Sequence[a]	Mol wt[b]	MIC (μg mL^{-1})[c]
Y-(ACHC-V-K)$_3$	β^{3}hTyr-(ACHC-β^{3}hVal-β^{3}hLys)$_3$	1791.9	8–16
Y-(ACHC-ACHC-K)$_3$	β^{3}hTyr-(ACHC-ACHC-β^{3}hLys)$_3$	1827.9	16–32
Y-(V-V-K)$_3$	β^{3}hTyr-(β^{3}hVal-β^{3}hVal-β^{3}hLys)$_3$	1755.9	32–64
Y-(ACHC-F-K)$_3$	β^{3}hTyr-(ACHC-β^{3}hPhe-β^{3}hLys)$_3$	1936.0	8
ent-Y-(ACHC-F-K)$_3$	(*R*-β^{3}hTyr)-[(*R*,*R*-ACHC)-(*R*-β^{3}hPhe)-(*R*-β^{3}hLys)]$_3$	1936.0	8
Y-(ACHC-K-K)$_3$	β^{3}hTyr-(ACHC-β^{3}hLys-β^{3}hLys)$_3$	2221.1	128–>128
(ACHC-V-K)$_3$	(ACHC-β^{3}hVal-β^{3}hLys)$_3$	1614.7	16–32
Scrambled Y-(ACHC-ACHC-K)$_3$	β^{3}hTyr-ACHC-ACHC-(β^{3}hLys-ACHC)$_3$-ACHC	1827.9	>128
Scrambled (ACHC-V-K)$_3$	β^{3}hLys-β^{3}hVal-β^{3}hVal-ACHC-β^{3}hLys-β^{3}hVal-ACHC-ACHC-β^{3}hLys	1614.7	>128
K7E Y-(ACHC-ACHC-K)$_3$	β^{3}hTyr-(ACHC-ACHC-β^{3}hLys)-(ACHC-ACHC-β^{3}hGlu)-(ACHC-ACHC-β^{3}hLys)	1714.9	>128
K10E Y-(ACHC-ACHC-K)$_3$	β^{3}hTyr-(ACHC-ACHC-β^{3}hLys)$_2$-(ACHC-ACHC-β^{3}hGlu)	1714.9	>128
Y-(ACHC-ACHC-E)$_3$	β^{3}hTyr-(ACHC-ACHC-β^{3}hGlu)$_3$	1488.7	>128[d]
C2-Y-(ACHC-ACHC-K)$_3$	Acetyl-β^{3}hTyr-(ACHC-ACHC-β^{3}hLys)$_3$	1755.9	32–64
C6-Y-(ACHC-ACHC-K)$_3$	Hexanoyl-β^{3}hTyr-(ACHC-ACHC-β^{3}hLys)$_3$	1812.1	16–64
C9-Y-(ACHC-ACHC-K)$_3$	Nonanoyl-β^{3}hTyr-(ACHC-ACHC-β^{3}hLys)$_3$	1854.1	16
C15-Y-(ACHC-ACHC-K)$_3$	Pentadecanoyl-β^{3}hTyr-(ACHC-ACHC-β^{3}hLys)$_3$	1938.3	>128
Coumarin-(ACHC-V-K)$_3$	7-Methoxycoumarin-(ACHC-β^{3}hVal-β^{3}hLys)$_3$[e]	1702.8	16–64
Coumarin-linker-(ACHC-V-K)$_3$	7-Methoxycoumarin-(ethylene glycol)$_2$-(ACHC-β^{3}hVal-β^{3}hLys)$_3$[e]	1848.0	16–32

[a]The C-terminus is amidated for all β-peptides. β-Peptide residues are *S*-enantiomers, except where noted. [b]Molecular weights include trifluoroacetic acid counterions. [c]Planktonic *C. albicans* cells (10^3 cells mL^{-1}) were incubated with 2-fold dilutions of β-peptides for 48 h to determine MICs. Data are the MIC range obtained for testing activity against SC5314, ATCC 24433, and ATCC 90028 in at least two independent experiments with three replicates each. Individual MIC ranges for the three strains are provided in Supplementary Table S1. [d]MIC data are for activity against SC5314 only. [e]Structures for N-terminal 7-methoxycoumarin and (ethylene glycol)$_2$ appendages are provided in Supporting Information.

erties of side chain arrays presented by the β-peptide 14-helix.

Activity against Planktonic *C. albicans*. We tested the susceptibility of planktonic *C. albicans* to β-peptides with different sequences using a modified version of the procedure recommended by the Clinical Laboratory Standards Institute (*28*). In addition to visually determining the MICs (Table 1), we used a quantitative XTT reduction assay to obtain the concentration dependence of growth inhibition due to the β-peptides (Figure 2). Because similar results were obtained for the three strains tested (SC5314, ATCC 24433, and ATCC 90028), combined results are shown in Table 1 and Figure 2. Data for individual strains are provided in Supporting Information. Y-(ACHC-F-K)$_3$, Y-(ACHC-V-K)$_3$, Y-(ACHC-ACHC-K)$_3$, and Y-(V-V-K)$_3$ were designed to be globally amphiphilic in the 14-helical conformation, and these compounds prevented growth of 100% of planktonic *C. albicans* at concentrations as low as 8 μg mL^{-1} (Figure 2, panel a). When compared on a molar basis, the MICs of Y-(ACHC-F-K)$_3$ (4.1 μM), Y-(ACHC-V-K)$_3$ (4.5–8.9 μM), and Y-(ACHC-ACHC-K)$_3$ (8.8–17.5 μM) are only slightly higher than the MIC of amphotericin B (~1.1 μM) but are an order of magnitude higher than MICs reported for the relatively new antifungal drug caspofungin (0.012–0.82 μM) (*29*). The MICs of the β-peptides are comparable to or better than MICs for fluconazole (0.4–26.1 μM for strains lacking fluconazole resistance) (*30*) and the broad spectrum, membrane-active antiseptic chlorhexidine (7.0–13.9 μM) (*31, 32*).

The "scrambled" isomer of Y-(ACHC-V-K)$_3$, which is not globally amphiphilic in the 14-helical conformation, did not significantly reduce cell growth at concentrations up to 128 μg mL^{-1}. This observation is consistent with results previously reported for antibacterial and antifungal β-peptides (*17, 27, 33, 34*). The requirement of a globally amphiphilic helix for antifungal activity is expected, since the proposed mechanisms for antimicro-

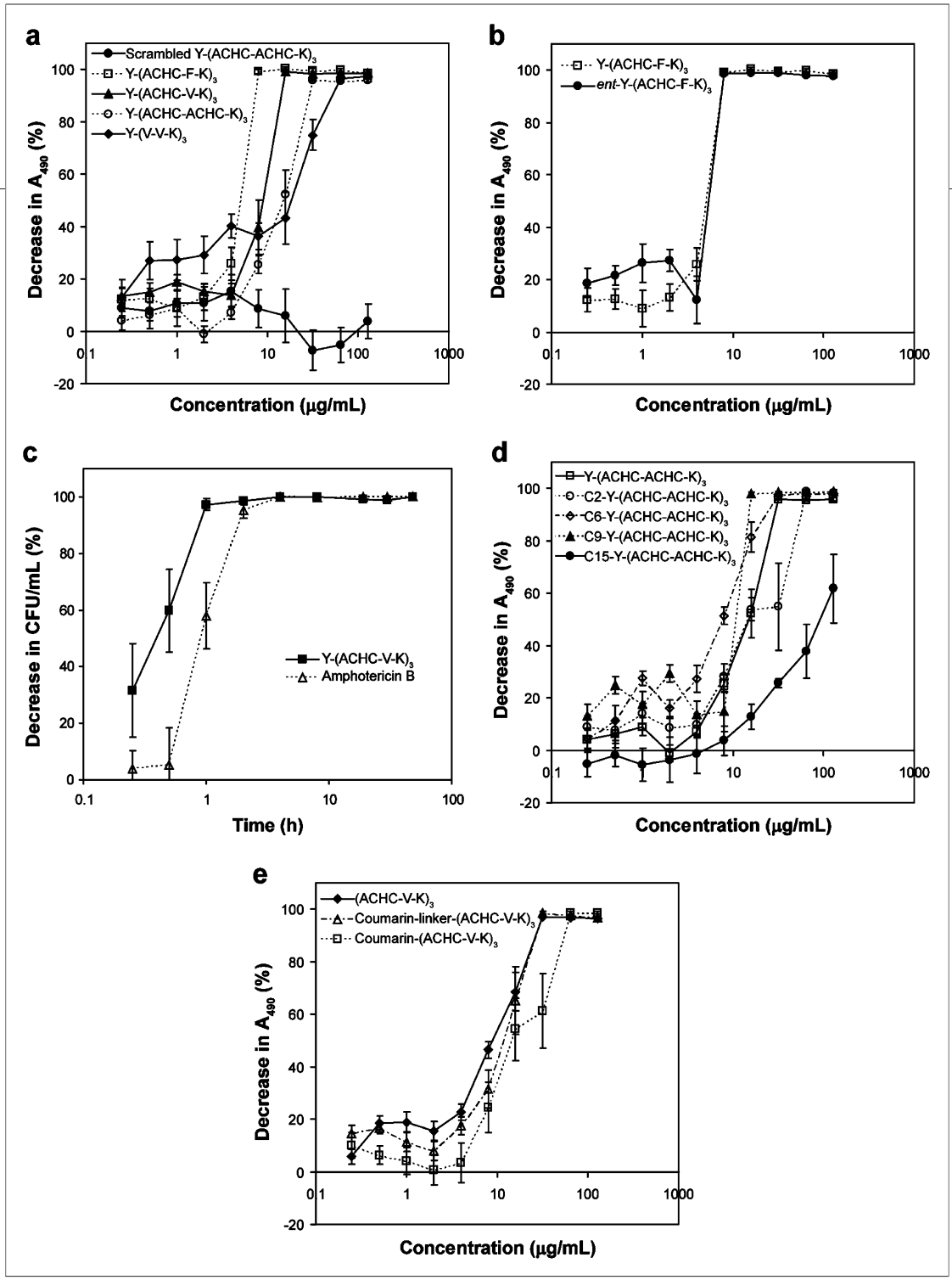

Figure 2. Susceptibility of planktonic *C. albicans* cells to β-peptides. a) Activity of β-peptides with different sequences. b) Activity of β-peptide enantiomers. c) Kinetics of planktonic activity of Y-(ACHC-V-K)$_3$ and amphotericin B. d) Activity of β-peptides with N-terminal acylation. e) Activity of β-peptides with N-terminal 7-methoxycoumarin fluorescent label attached directly and *via* an ethylene glycol linker. *C. albicans* cells (10^3 cells mL^{-1}) were incubated with β-peptides for 48 h. For panels a, b, d, and e, susceptibility was assessed using an XTT reduction assay to compare the absorbance at 490 nm for β-peptide-treated samples and untreated samples. Data points are averages for testing three different strains (ATCC 24433, ATCC 90028, and SC5314) in at least two independent experiments. The variations among strains were minimal. Individual data for each strain are shown in Supplementary Figures S1 and S2. Error bars represent standard error of the mean. For panel c, *C. albicans* strain SC5314 cells (10^3 cells mL^{-1}) were incubated with β-peptide at 32 μg mL^{-1} or amphotericin B at 2 μg mL^{-1} (twice the planktonic MICs). At each time point, treated and untreated samples were plated, and colony forming units (CFUs) were counted to calculate the decrease in CFUs due to the antifungal agents. Results are averages of five independent experiments, and error bars represent standard error of the mean.

TABLE 2. Effect of net charge on β-peptide activity against planktonic *C. albicans*

β-Peptide	Charge	MIC (μg mL^{-1})[a]
Y-(ACHC-K-K)$_3$	+7	128–>128
Y-(ACHC-ACHC-K)$_3$	+4	16–32
C2-Y-(ACHC-ACHC-K)$_3$	+3	32
K7E Y-(ACHC-ACHC-K)$_3$	+2	>128
K10E Y-(ACHC-ACHC-K)$_3$	+2	>128
Y-(ACHC-ACHC-E)$_3$	−2	>128

[a]Planktonic *C. albicans* SC5314 cells (10^3 cells mL^{-1}) were incubated with 2-fold dilutions of β-peptides for 48 h to determine MICs. Data are the MIC range obtained in at least two independent experiments with three replicates each.

bial peptide activity require segregation of charged and hydrophobic residues upon folding in order to interact with microbial membranes (*6, 7*).

If the mechanism of action of the β-peptides were to involve binding to a specific protein or other cellular component, then changing the absolute configuration of the β-peptide would be expected to affect activity. However, *ent*-Y-(ACHC-F-K)$_3$ displayed activity nearly identical to that of its enantiomer Y-(ACHC-F-K)$_3$ (Figure 2, panel b). Indifference of antibacterial activity to absolute configuration has been reported previously for both α- and β-peptides (*8, 17, 35*), and this indifference is consistent with a mechanism of action that involves membrane disruption through nonspecific interactions.

To evaluate the effect of net charge on β-peptide antifungal activity, we examined a series of β-peptides with net charges expected to range from −2 to +7 at neutral pH (Table 2). This series is based on Y-(ACHC-ACHC-K)$_3$, which has an expected net charge of +4 (three cationic side chains and a cationic N-terminus). We increased the charge by substituting one of the ACHC residues in the repeating unit with a positively charged β^{3}hLys residue. We reduced the charge by acetylating the N-terminus or by substituting one or more β^{3}hLys residues with negatively charged β^{3}hGlu residues. Increasing the net charge to +7 drastically reduced activity, raising the MIC to 128 μg mL^{-1} or greater. This effect may reflect the decreased hydrophobicity of Y-(ACHC-K-K)$_3$ relative to Y-(ACHC-ACHC-K)$_3$, which could diminish the membrane disruption capabilities of the β-peptide. Prior work with a different family of β-peptides demonstrated a comparable loss of activity against bacteria with increased net charge (*33, 34*). Reducing the net charge by 1 (from +4 to +3) through acetylation of the N-terminus caused little change in the MIC. However, when the charge was further reduced to +2 or −2 by replacing β^{3}hLys with β^{3}hGlu, the activity was significantly reduced (MIC > 128 μg mL^{-1}). The loss of activity upon incorporation of the anionic side chain was likely caused by diminished electrostatic attraction of the β-peptide to the anionic cell membrane.

Kinetics of Planktonic Toxicity. Antimicrobial α-peptides typically kill planktonic bacteria and fungi within several minutes to several hours (*36–38*). The kinetics of planktonic toxicity for Y-(ACHC-V-K)$_3$ were evaluated and compared to the kinetics of amphotericin B toxicity at twice the MIC of each compound (32 μg mL^{-1} for Y-(ACHC-V-K)$_3$ and 2 μg mL^{-1} for amphotericin B). As Figure 2, panel c shows, Y-(ACHC-V-K)$_3$ killed over 50% of *C. albicans* cells within 30 min. Y-(ACHC-V-K)$_3$ completely eliminated growth by 4 h, the same time required for complete killing with amphotericin B.

Hemolytic Activity. We evaluated the toxicity of the β-peptides toward mammalian cells by measuring their ability to lyse red blood cells (Figure 3). The antifungal β-peptides (ACHC-V-K)$_3$, Y-(ACHC-ACHC-K)$_3$, and Y-(V-V-K)$_3$ exhibited less hemolytic activity than did a derivative of the α-peptide magainin 2. These β-peptides displayed low levels of hemolysis at their MICs, while Y-(ACHC-F-K)$_3$ and Y-(ACHC-V-K)$_3$ showed levels of hemolysis significantly higher than that of the magainin 2 derivative. As with antifungal activity, global amphiphilicity played an important role in hemolytic activity. The globally amphiphilic β-peptide Y-(ACHC-ACHC-K)$_3$ showed a relatively low level of hemolysis compared to that of other β-peptides and the magainin 2 derivative, but consistent with prior reports (*17, 33*), the nonglobally amphiphilic scrambled isomer of Y-(ACHC-ACHC-K)$_3$ showed essentially no hemolytic activity. Removal of the N-terminal β^{3}hTyr residue from Y-(ACHC-V-K)$_3$ substantially decreased the hemolytic activity but caused only a small reduction in antifungal activity (Figure 3 and Table 1). Although the mechanisms of β-peptide selectivity are still poorly understood, our results show that β-peptides can be designed for selective toxicity toward fungal cells over red blood cells.

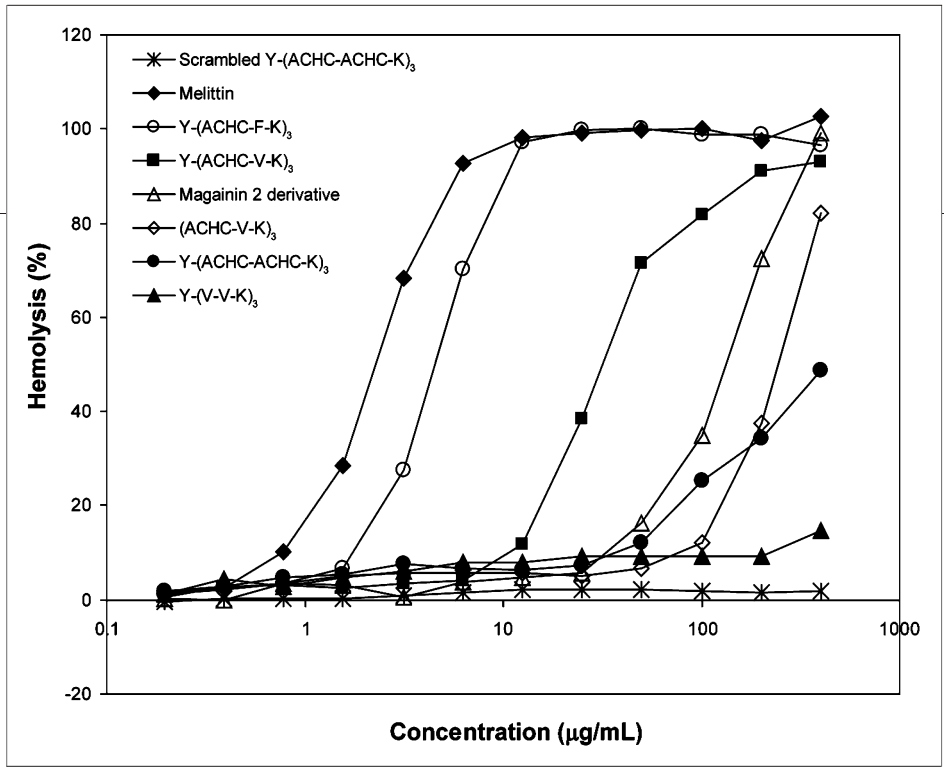

Figure 3. Hemolytic activity of β-peptides. Two-fold dilutions of β-peptides were incubated with human red blood cells for 1 h, and the absorbance of the supernatant was measured at 405 nm to calculate the percent of red blood cells lysed. Results for (Ala[8,13,18])-magainin 2 amide, a magainin 2 derivative, and for melittin, an α-peptide used as the 100% hemolysis control, are included for comparison.

β-Peptide Acylation. Makovitzki *et al.* (*39*) have reported that N-terminal acylation of short cationic peptides with fatty acids can enhance antibacterial and antifungal activity, so we evaluated the effect of acylation on β-peptide toxicity toward *C. albicans*. We capped the N-terminus of Y-(ACHC-ACHC-K)$_3$ with acetyl, hexanoyl, nonanoyl, and pentadecanoyl groups (Figure 2, panel d). Attaching acetyl, hexanoyl, or nonanoyl groups had relatively little effect on antifungal activity. In contrast, the pentadecanoyl group substantially reduced antifungal activity relative to that of Y-(ACHC-ACHC-K)$_3$. The long acyl tail may cause the β-peptide to aggregate, which could interfere with the interaction between the β-peptide and *C. albicans* membranes.

Fluorescently Labeled β-Peptides. To visualize the interaction of β-peptides with *C. albicans* cells, we attached a fluorophore to (ACHC-V-K)$_3$, a β-peptide with strong antifungal activity and low hemolytic activity at the antifungal MIC (*27*). Previous reports of fluorescently labeled antimicrobial peptides are sparse, with the label limited mainly to a fluorescein derivative (*9, 10, 40*). Fluorescein labeling eliminated antifungal β-peptide activity (see Supplementary Figure S5), making it unsuitable for our localization studies. Because our N-terminal capping studies suggested that a small, uncharged appendage would not compromise the activity of the β-peptide, we attached 7-methoxycoumarin-3-carboxylic acid to the N-terminus of (ACHC-V-K)$_3$. When directly attached to the N-terminus, the fluorophore caused a modest reduction of the β-peptide antifungal activity (Figure 2, panel e). Use of a {2-[2-(amino)-ethoxy]-ethoxy}-acetyl (AEEA) spacer between the N-terminus and the fluorophore produced a labeled β-peptide with activity that was indistinguishable from that of (ACHC-V-K)$_3$ itself (Figure 2, panel e). In addition to 7-methoxycoumarin, we labeled (ACHC-V-K)$_3$ with 7-nitrobenz-2-oxa-1,3-diazole (NBD), another small, uncharged label, but NBD conjugation diminished antifungal activity (see Supplementary Figure S5). The structures of the fluorophores and the ethylene glycol linker are provided in the Supporting Information.

We used confocal microscopy to observe the interaction of coumarin-linker-(ACHC-V-K)$_3$ with *C. albicans*. The β-peptide was observed throughout the cytoplasm of the *C. albicans* cells (Figure 4, panel a). Cells containing the β-peptide did not show the red cylindrical intravacuolar structures (CIVS) characteristic of metabolically active cells, indicating that these cells were not viable. Conversely, cells containing the brightly stained red CIVS expected in viable cells did not contain detectable levels of β-peptide (Figure 4, panels b–f). When the concentration of β-peptide was decreased, a corresponding decrease in the ratio of dead cells (containing β-peptide but not CIVS) to live cells (containing CIVS but not β-peptide) was observed (Figure 4, panels b–f).

Previous studies have shown that different cationic antimicrobial peptides can have distinctive localization within target cells. Some peptides associate only with the cell surface (*9, 41*), while other peptides penetrate the cell membrane and accumulate in the cytoplasm (*40, 41*) or associate with particular organelles (*10*). Work with model membranes corroborates the observation that the localization varies among peptides and implies that although membrane interaction is a key step in killing microbes, the ability to cross the cell membrane and reach intracellular targets may also be important for some peptides (*42*). The exact mechanism of the antifungal effects exerted by our β-peptides is still not known, but the accumulation of coumarin-linker-

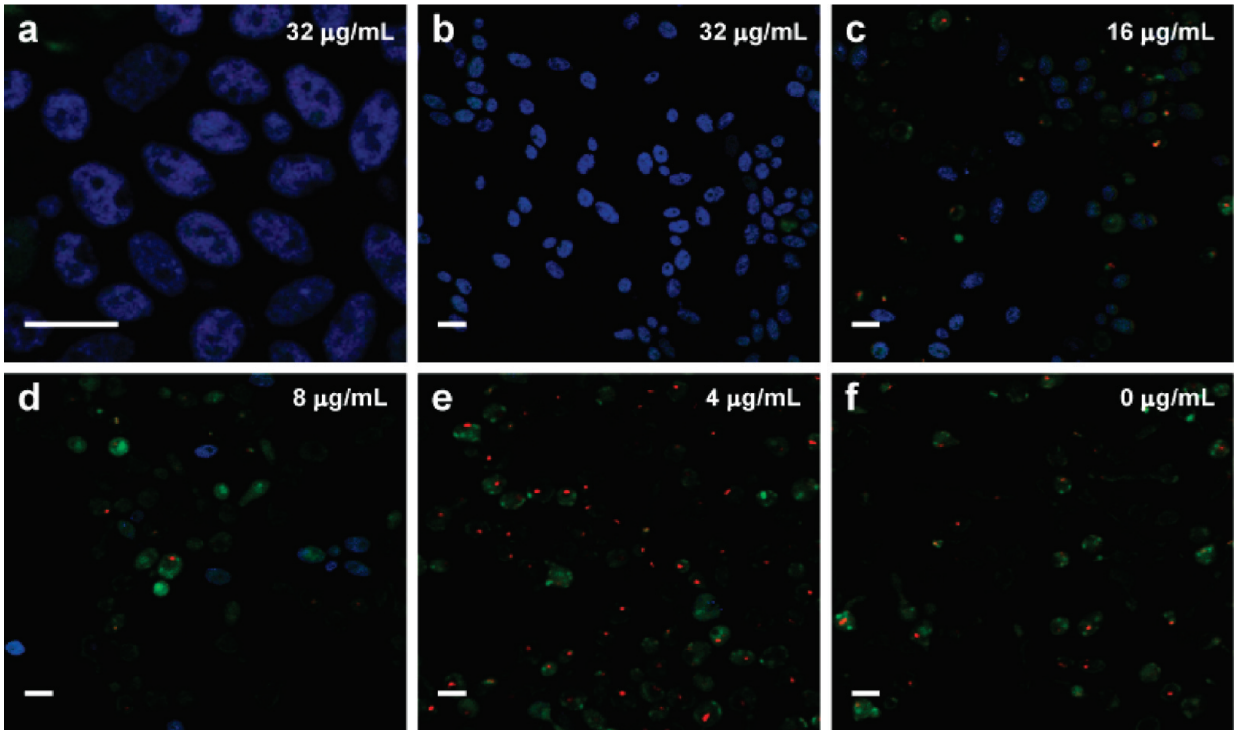

Figure 4. Confocal microscopy of *C. albicans* cells treated with coumarin-linker-(ACHC-V-K)$_3$. *C. albicans* strain ATCC 24433 cells (0.5×10^7 cells mL^{-1}) were treated with the labeled β-peptide (blue) at the indicated concentrations for 2.5 h. Cells were stained with FUN-1, which is converted from a diffuse green dye to condensed red CIVS in metabolically active cells. Scale bars = 5 μm.

(ACHC-V-K)$_3$ in the cytoplasm of metabolically inactive cells suggests β-peptide-mediated disruption of the cell membrane. Permeabilization of the membrane by β-peptides was confirmed using a propidium iodide permeability assay (data provided in Supplementary Table S2). The presence of detectable levels of labeled β-peptide only in dead cells raises the possibility that β-peptide-induced killing involves some sort of cooperative phenomenon. Once a minimum level of membrane damage has been achieved for a given cell, β-peptides may be attracted to the now-accessible cell interior in preference to intact cells nearby. Our observations suggest that β-peptides do not attain lethality by targeting a specific organelle within *C. albicans* cells, but the results do not rule out a contribution from nonspecific interactions with cytoplasmic components.

Activity against *C. albicans* Biofilms. In addition to evaluating the activity of β-peptides against planktonic cells, we evaluated their activity against biofilms formed for 48 h in 96-well plates (Figure 5, panel a). All β-peptides that were lethal to planktonic cells were also able to kill biofilms, but concentrations of 1–16 times the planktonic MICs were needed to achieve 80% reduction in metabolic activity of the biofilms. At concentrations of 128–256 μg mL^{-1}, no metabolic activity was detected in the biofilms treated with active β-peptides. Scrambled (ACHC-V-K)$_3$, a β-peptide that cannot form a globally amphiphilic 14-helix and lacks activity in the planktonic assay (*27*), did not achieve 80% reduction in metabolic activity of biofilms at 512 μg mL^{-1}, the highest concentration tested. In contrast to the activity against planktonic cells, the activity against biofilms did not show distinct differences among globally amphiphilic β-peptides with different sequences. For example, Y-(ACHC-F-K)$_3$ had an MIC of 8 μg mL^{-1} and Y-(V-V-K)$_3$ had an MIC of 64 μg mL^{-1} in the planktonic assay (Figure 2, panel a), but they both eliminated biofilm metabolic activity at 256 μg mL^{-1} and produced similar metabolic activity reduction at lower concentrations (Figure 5, panel a).

We used coumarin-linker-(ACHC-V-K)$_3$ to probe the interaction of β-peptides with *C. albicans* biofilms. The

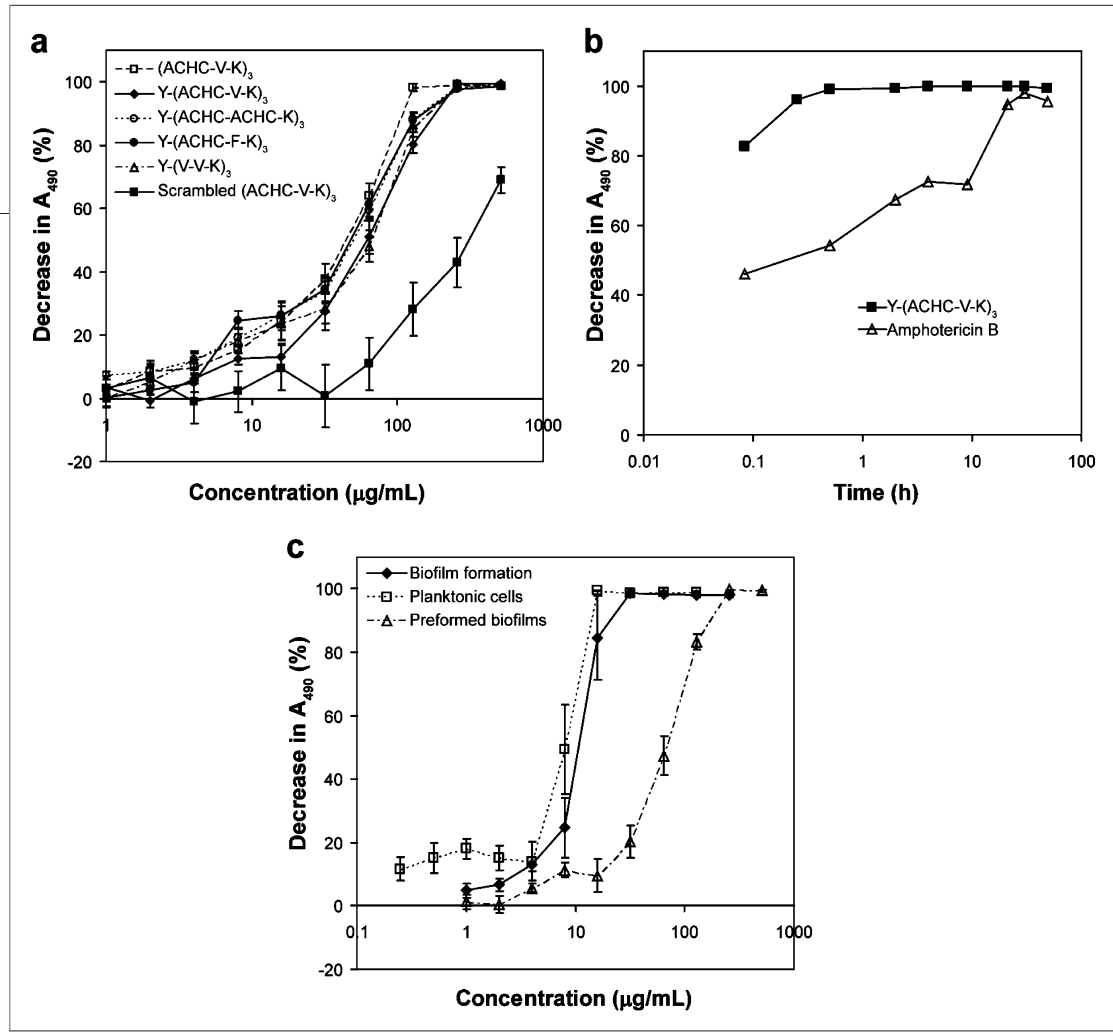

Figure 5. Activity of β-peptides against *C. albicans* biofilms. a) Susceptibility of preformed *C. albicans* biofilms to β-peptides with different sequences. Biofilms were incubated for 48 h with β-peptides, and susceptibility was assessed using an XTT reduction assay to compare the absorbance at 490 nm for β-peptide-treated samples and untreated samples. Results are averages for biofilms formed by three strains (ATCC 24433, ATCC 90028, and SC5314) in at least two independent experiments. Error bars represent standard error of the mean. Individual data for each strain are shown in Supplementary Figure S3. b) Kinetics of biofilm growth inhibition for Y-(ACHC-V-K)$_3$ and amphotericin B. *C. albicans* strain SC5314 biofilms were incubated with β-peptide at 512 μg mL^{-1} or amphotericin B at 8 μg mL^{-1} (twice the concentrations needed for 100% growth inhibition of biofilms). At each time point, an XTT reduction assay was used to compare antifungal-treated biofilms to untreated biofilms. Results are averages of two independent experiments. c) Prevention of biofilm formation. *C. albicans* cells (10^6 cells mL^{-1}) were incubated with Y-(ACHC-V-K)$_3$ for 48 h to allow biofilm formation. An XTT reduction assay was used to evaluate growth of biofilms in the presence of β-peptide compared to growth of biofilms with no β-peptide present. Results are averages for testing two strains (SC5314 and ATCC 90028) in at least two separate experiments. Error bars represent standard error of the mean. Data for planktonic susceptibility and preformed biofilm susceptibility are included for comparison.

β-peptide-treated biofilm was less dense than an untreated biofilm (Figure 6, panels a–d), and the β-peptide was detected in both yeast and hyphal cells throughout the depth of the biofilm (Figure 6, panels c–f). Similar to the results for planktonic cells, biofilm-associated yeast and hyphae containing the labeled β-peptide did not contain the CIVS indicative of live, metabolically active cells. These observations are consistent with the lack of metabolic activity found in biofilms treated with sufficient concentrations of β-peptide during biofilm susceptibility testing.

As expected on the basis of prior work with antimicrobial peptides (*37, 43*) and small-molecule antifungal agents (*3*), the β-peptides required higher concentrations to inhibit growth of biofilms than to kill planktonic cells. The higher concentration requirement may arise from depletion of β-peptide by the large number of cells present in biofilms, from resistance mechanisms displayed by cells within biofilms, or from a combination of these effects. Resistance of planktonic bacteria and *C. albicans* to antimicrobial peptides has been reported through mechanisms involving cell surface changes, in-

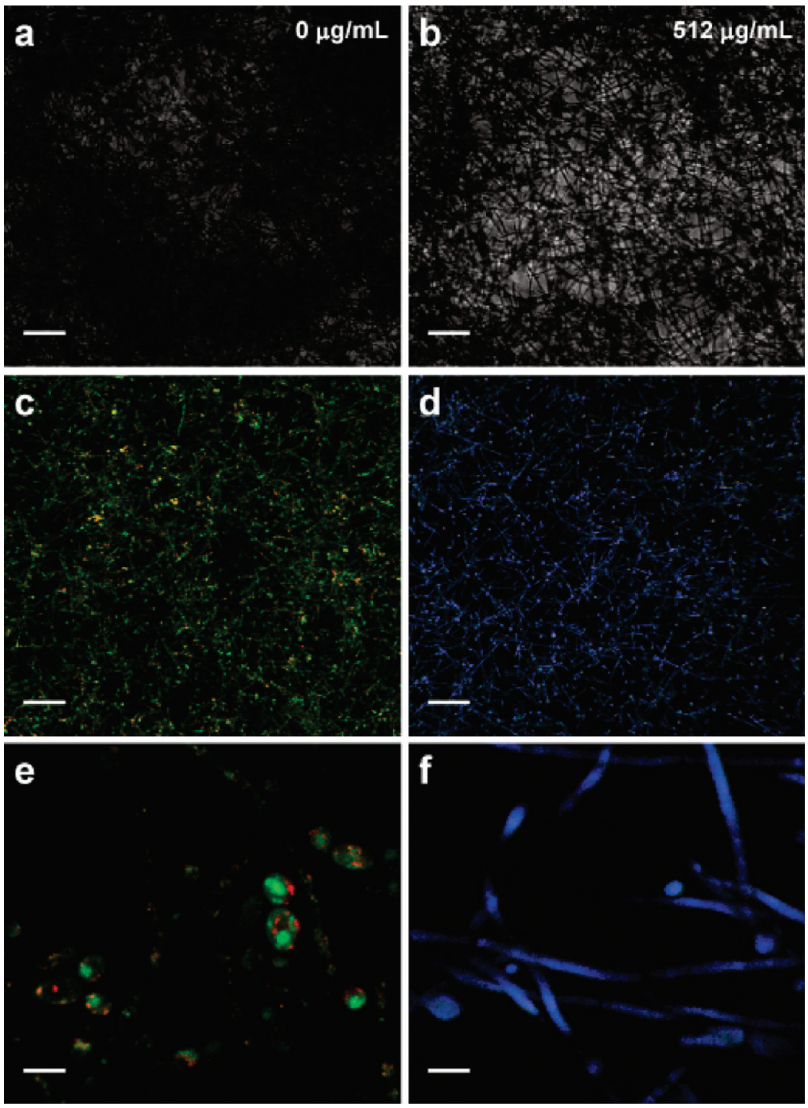

Figure 6. Microscopy images of *C. albicans* biofilms treated with coumarin-linker-(ACHC-V-K)$_3$ using transmission (a, b) and confocal (c–f) microscopy. *C. albicans* strain SC5314 biofilms were formed in glass-bottomed dishes and incubated for 48 h with 0 μg mL^{-1} (panels a, c, e) or 512 μg mL^{-1} (panels b, d, f) of labeled β-peptide (blue). Biofilms were stained with FUN-1, which is converted from a diffuse green dye to condensed red CIVS in metabolically active cells. The confocal planes in images c and d are near the base of the biofilms. Scale bars = 50 μm for panels a–d. Scale bars = 5 μm for panels e and f.

cluding decreased negative charge density and decreased membrane fluidity (*44*, *45*).

Kinetics of Biofilm Growth Inhibition. We evaluated the kinetics of biofilm growth inhibition by calculating the reduction in metabolic activity of *C. albicans* strain SC5314 biofilms after treatment with amphotericin B or Y-(ACHC-V-K)$_3$ at twice the concentration required for 100% biofilm growth inhibition after 48 h (8 μg mL^{-1} for amphotericin B and 512 μg mL^{-1} for Y-(ACHC-V-K)$_3$). Y-(ACHC-V-K)$_3$ completely inhibited biofilm metabolic activity in less than 1 h (Figure 5, panel b), whereas amphotericin B required considerably longer, approximately 30 h, to reach its maximum lethality.

Effect of β-Peptide on Biofilm Formation. We assessed the ability of Y-(ACHC-V-K)$_3$ to inhibit the formation of *C. albicans* biofilms by including 2-fold dilutions of this β-peptide during biofilm formation. After 48 h, we used an XTT reduction assay to quantify biofilm growth. As Figure 5, panel c shows, the β-peptide was able to kill *C. albicans* cells and completely inhibit biofilm formation at a β-peptide concentration very similar to the MIC measured in the planktonic growth assay (Table 1), even though the cell concentration in the biofilm formation assay was about 1000 times the cell concentration in the planktonic assay. The concentration needed to prevent biofilm formation was 4-fold less than the concentration needed to kill preformed biofilms (Figure 5, panel a).

These results suggest that antifungal β-peptides could be used to prevent colonization and biofilm formation by *C. albicans*, with planktonic lethality

achieved at much lower concentrations than those required to kill established biofilms. The β-peptides could be incorporated into surfaces using methods previously established for antimicrobial peptides and peptide mimics, such as covalent attachment (*46–48*). Since the β-peptide Y-(ACHC-ACHC-K)$_3$ maintained activity after N-terminal acylation with a variety of groups, this β-peptide also might maintain activity after covalent conjugation to a surface. Covalent attachment would be desirable for biomedical device surfaces relative to noncovalent association with the surface, because covalent attachment could generate a high local β-peptide concentration without systemic β-peptide exposure. 14-Helical β-peptides such as Y-(ACHC-ACHC-K)$_3$ are active against Gram-positive and Gram-negative bacteria (*17*), suggesting that β-peptides in this class are promising candidates for preventing colonization by a broad spectrum of fungal and bacterial pathogens.

Conclusions. The results reported here significantly extend the understanding of the antifungal effects exerted by designed β-peptides. Previous structure–activity studies showed that several β-peptides have activity against planktonic *C. albicans* (*27*). Findings reported here indicate β-peptides do not merely inhibit *C. albicans* growth but also rapidly kill planktonic cells. Results with sequence-scrambled and amphiphilic isomers and results with enantiomers support the hypothesis that β-peptides act by disrupting the cell membrane of fungal cells. We also showed that β-peptides inhibit *C. albicans* growth in established biofilms but that this inhibition requires higher concentrations than those needed for planktonic growth inhibition. In contrast, β-peptides active against planktonic *C. albicans* prevented biofilm formation at concentrations similar to those required for planktonic growth inhibition. After identifying a fluorescent labeling scheme that did not significantly alter antifungal activity, we examined the interactions of the labeled β-peptide with *C. albicans* planktonic cells and biofilms. The labeled β-peptide was found intracellularly in dead cells, but live cells did not contain detectable levels of β-peptide, which provides further evidence for a membrane disruption mechanism.

METHODS

β-Peptide Synthesis. β-Peptides were synthesized in a microwave reactor *via* Fmoc solid-phase synthesis methods using *O*-benzo-triazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBT), and *N*,*N*-diidopropylethylamine (DIEA) in *N*,*N*-dimethylformamide (DMF) for coupling reactions and piperidine in DMF for deprotection, as described previously (*27, 49*). For sequences containing a fluorescent group, the ethylene glycol linker {2-[2-(Fmoc-amino)-ethoxy]-ethoxy}-acetic acid (Bachem) and 7-methoxycoumarin-3-carboxylic acid (Molecular Probes) were coupled at RT using the same reagents. Acylation was achieved by coupling the appropriate carboxylic acid in the microwave with HBTU, HOBT, and DIEA or by coupling the respective symmetric anhydride at RT in a methylene chloride solution containing triethylamine. β-Peptides were cleaved from the resin and side chains were deprotected using a solution containing trifluoroacetic acid (95% v/v), triisopropylsilane (2.5%), and water (2.5%). After purification by reversed-phase high-pressure liquid chromatography (HPLC), β-peptide molecular weights were determined *via* matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy. HPLC and mass spectroscopy data are provided in Supporting Information.

Planktonic Susceptibility Testing. Susceptibility of planktonic *C. albicans* cells to β-peptides was tested using a broth microdilution method (*28*). Two-fold dilutions of amphotericin B and β-peptides were prepared in RPMI 1640 (pH 7.0, with L-glutamine and phenol red, buffered with 3-(*N*-morpholino) propanesulfonic acid), and 100 μL was mixed with 100 μL of cell suspensions of ATCC 90028, ATCC 24433, and SC5314 *C. albicans* strains in RPMI 1640 ($1–5 \times 10^3$ cells mL^{-1}) in 96-well plates. Antifungal agent-free medium controls and medium sterility controls were also included. Plates were incubated at 35 °C for 48 h, and growth was assessed.

After visually determining MICs, 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) was used to obtain quantitative results. Each well received 100μL of XTT solution (0.5 g L^{-1}, 3 μM menadione) in phosphate-buffered saline (PBS, pH 7.4), and the plates were incubated at 37 °C for 1.5 h. After centrifugation of the plates, the supernatant was transferred to a new plate, and the absorbance at 490 nm was measured. The decrease in metabolic activity of each strain due to the addition of amphotericin B or β-peptide was calculated from

$$\%\ \text{decrease in } A_{490} = 100 \times \frac{(A_{490}^{\text{untreated}} - A_{490}^{\text{background}}) - (A_{490} - A_{490}^{\text{background}})}{A_{490}^{\text{untreated}} - A_{490}^{\text{background}}}$$

where A_{490} is the average absorbance for wells containing a specified concentration of antifungal agent and a specified *C. albicans* strain, $A_{490}^{\text{untreated}}$ is the average absorbance for wells with a specified strain not treated with an antifungal agent, and $A_{490}^{\text{background}}$ is the average absorbance for wells containing only medium and XTT. At least three replicates for each antifungal agent/strain combination were included, and testing was performed on at least two different days.

Planktonic Growth Inhibition Kinetic Studies. A cell suspension of SC5314 was prepared in the same manner as for planktonic susceptibility testing. Solutions of Y-(ACHC-V-K)$_3$ and amphotericin B were prepared in RPMI 1640 at four times their planktonic MICs, and the antifungal agent solutions and antifungal agent-free controls were put in 96-well plates (100 μL per

well). An equal volume of the cell suspension was added to give final concentrations of 32 μg mL^{-1} for Y-(ACHC-V-K)$_3$ and 2 μg mL^{-1} for amphotericin B, and the plates were incubated at 35 °C. At predetermined times, solution from wells containing three replicates of β-peptide, amphotericin B, and the antifungal agent-free control were agitated by pipetting. Serial dilutions of the well contents were plated on yeast extract-peptone-dextrose (YPD) agar, and the plates were incubated at 30 °C for 24–48 h. Colony forming units (CFUs) were counted, and the reduction in growth due to the amphotericin B and the β-peptide was determined by comparing the number of CFUs in samples from wells containing antifungal agent with the number of CFUs in samples from untreated wells.

Hemolysis Testing. Lysis of human red blood cells by β-peptides was measured as described previously (*17*, *34*). After washing red blood cells with Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 7.2), the red blood cells were resuspended in TBS at a concentration of 1% (v/v). The suspension (80 μL) was mixed with 2-fold dilutions of β-peptide or α-peptide (20 μL) in 96-well plates. Wells containing TBS provided a negative lysis control, and wells containing a high concentration of the α-peptide melittin (Sigma) provided a positive lysis control. The α-peptide (Ala^{8,13,18})-magainin 2 amide (Sigma), a magainin 2 derivative, was also included. Plates were incubated for 1 h at 37 °C, and following centrifugation, 50 μL of the supernatant was put in a fresh plate with an equal volume of water. The absorbance of the supernatant at 405 nm was measured to detect hemoglobin released by lysed red blood cells. The percent hemolysis was calculated from

$$\%\ \text{hemolysis} = \frac{A_{405} - A_{405}^{\text{TBS}}}{A_{405}^{\text{complete lysis}} - A_{405}^{\text{TBS}}} \cdot 100$$

where A_{405} is the absorbance of the supernatant from red blood cells treated with peptide, A_{405}^{TBS} is the absorbance of the supernatant from red blood cells in TBS, and $A_{405}^{\text{complete lysis}}$ is the average absorbance of the supernatant from red blood cells treated with melittin at concentrations of 50–400 μg mL^{-1}. Two replicates were included in each experiment, and experiments were performed at least two times.

Biofilm Susceptibility Testing. Testing of the susceptibility of *C. albicans* biofilms to β-peptide treatment was based on the method described by Ramage and Lopez-Ribot (*50*). SC5314, ATCC 24433, and ATCC 90028 strains were grown in liquid YPD medium overnight at 30 °C. The cells were washed with PBS and resuspended in RPMI 1640. A cell suspension of 1 × 10^6 cells mL^{-1} in RPMI 1640 was added to wells of 96-well plates (100 μL per well). Plates were incubated at 37 °C for 48 h to allow biofilm formation.

After formation, biofilms were washed with PBS to remove nonadherent cells. Two-fold serial dilutions of β-peptides and amphotericin B in RPMI 1640 were added to the biofilms (100 μL per well). Positive growth controls (RPMI 1640 only) were included. Plates were incubated at 37 °C for an additional 48 h and washed with PBS.

To quantify the reduction in metabolic activity, 100 μL of XTT solution (0.5 g L^{-1} XTT in PBS, 1 μM menadione) was added to biofilms and to background control wells. Plates were incubated for 1.5 h at 37 °C, and the XTT solution from each well was transferred into a new plate. The absorbance at 490 nm was measured, and the reduction in metabolic activity at each concentration was calculated from the same equation used for planktonic susceptibility testing. At least three replicates were included for each antifungal agent and *C. albicans* strain combination, and testing was performed on at least two different days.

Biofilm Growth Inhibition Kinetic Studies. SC5314 biofilms were formed in 96-well plates in the same manner as for biofilm susceptibility testing. After formation, biofilms were washed with PBS. Solutions containing Y-(ACHC-V-K)$_3$ or amphotericin B in RPMI 1640 at twice the concentration needed for 100% growth inhibition of biofilms (512 μg mL^{-1} for Y-(ACHC-V-K)$_3$ and 8 μg mL^{-1} for amphotericin B) and an antifungal agent-free control were added to biofilms (100 μL per well). At predetermined times, three replicates of biofilms treated with β-peptide, amphotericin B, and the antifungal agent-free control were washed, and the reduction in metabolic activity was determined with XTT.

Biofilm Formation in the Presence of β-Peptide. The ability of *C. albicans* to form biofilms in the presence of β-peptide was tested using a procedure modified from the protocol for testing biofilms preformed in 96-well plates. SC5314 and ATCC 90028 were grown in liquid YPD overnight at 30 °C, and the cells were washed with PBS. Cells were resuspended in RPMI 1640, and the concentration was adjusted to 2 × 10^6 cells mL^{-1} in RPMI 1640.

Y-(ACHC-V-K)$_3$ was serially diluted in RPMI 1640 and added to 96-well plates (50 μL per well), along with antifungal agent-free controls. At least three replicates were included. An equal volume of the cell suspension was added to the wells, giving a final cell concentration of 1 × 10^6 cells mL^{-1}, and the plates were incubated at 37 °C for 48 h.

After allowing biofilms to form for 48 h, wells were washed to remove nonadherent cells, and the reduction in metabolic activity for biofilms grown in the presence of β-peptide and amphotericin B was determined using XTT.

Imaging of Planktonic Cells and Biofilms. To image the interaction of coumarin-linker-(ACHC-V-K)$_3$ with *C. albicans* cells, a cell suspension of 1 × 10^7 cells mL^{-1} was prepared in RPMI 1640. Dilutions of coumarin-linker-(ACHC-V-K)$_3$ and a β-peptide-free control were prepared, and 7.5 mL of each solution was added to an equal volume of cell suspension. Cells were incubated with the solutions for 2.5 h at 35 °C, washed with PBS, and resuspended in RPMI 1640 containing FUN-1 (10 μM final concentration, Molecular Probes). After incubation for 1 h at 30 °C, cells were washed and resuspended in PBS with 2% glucose (PBS-glucose). Samples were pipetted onto 1% agarose disks to immobilize cells and covered with a no. 1.5 coverslip. The coverslip/disk assembly was inverted for microscopy.

Biofilms for imaging were prepared in essentially the same manner as biofilms for susceptibility testing. To facilitate imaging, biofilms were grown in glass-bottomed 35 mm dishes coated with poly-D-lysine (Mat-Tek), and volumes were scaled to account for the larger surface area. Biofilms were grown for 48 h and then incubated with coumarin-linker-(ACHC-V-K)$_3$ for 48 h. After washing the biofilms, a solution containing FUN-1 (10 μM) was added, and the biofilms were incubated with the dye for 30 min at 37 °C. Biofilms were washed, and fresh PBS-glucose was added to the dishes.

Fluorescence microscopy images were obtained with a Bio-Rad Radiance 2100 MP Rainbow confocal/multiphoton system. FUN-1 is converted from a diffuse green stain to compact red CIVS by metabolically active cells (*51*). The red and green fluorescence signals from FUN-1 were collected by exciting with lasers at 488 nm for the green signal (emission collected at 500–530 nm) and 543 nm for the red signal (emission collected at 570–625 nm). The 7-methoxycoumarin label was excited with a multiphoton laser at 720 nm, and the fluorescence signal was collected at wavelengths ≤490 nm. Images were processed with NIH ImageJ.

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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