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Silver Adducts of Four-Branched Histidine Rich Peptides Exhibit Synergistic Antifungal Activity

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Abstract

Previously, a four branched histidine-lysine rich peptide, H3K4b, was shown to demonstrate selective antifungal activity with minimal antibacterial activity. Due to the potential breakdown from proteases, H3K4b was further evaluated in the current study by varying the D- and L-amino acid content in its branches. Whereas analogues of H3K4b that selectively replaced L-amino acids (H3k4b, h3K4b) had improved antifungal activity, the all D-amino acid analogue, h3k4b, had reduced activity, suggesting that partial breakdown of the peptide may be necessary. Moreover, because histidines form coordination bonds with the silver ion, we examined whether silver adducts can be formed with these branched histidine-lysine peptides, which may improve antifungal activity. For *C. albicans*, the silver adduct of h3K4b or H3k4b reduced the MIC compared to peptide and silver ions alone by 4- and 5-fold, respectively. For *A. fumigatus*, the silver adducts showed even greater enhancement of activity. Although the silver adducts of H3k4b or h3K4b showed synergistic activity, the silver adduct with the all L-amino acid H3K4b surprisingly showed the greatest synergistic and growth inhibition of *A. fumigatus*: the silver adduct of H3K4b reduced the MIC compared to the peptide and silver ions alone by 30- and 26-fold, respectively. Consistent with these antifungal efficacy results, marked increases in free oxygen radicals were produced with the H3K4b and silver combination. These studies suggest that there is a balance between stability and breakdown for optimal antifungal activity of the peptide alone and for the peptide-silver adduct.

1. Introduction

The incidence of high mortality for invasive fungal infections is rising, and contributes to rising health care costs. Medical advances have created a burgeoning population of patients with severely compromised immune systems, which has elevated invasive fungal infection as an important cause of death in cancer patients and transplant recipients [1, 2]. *Candida*

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See Supplementary Data.

species now constitute the 4th most common pathogen isolated in all nosocomial bloodstream infections [3]. Cryptococcal meningitis caused by *Cryptococcus neoformans* and *Cryptococcus gattii* is the most common cause of fungal central nervous system infection in the world today, primarily affecting immunocompromised patients but also apparently normal hosts [4]. The incidence of invasive pulmonary aspergillosis (IPA) from *Aspergillus fumigatus* has increased 3-fold in the last decade with the current mortality from IPA ranging between 30 and 50% [5, 6].

Of the *Aspergillus* spp., *A. fumigatus* is the most common cause of invasive pulmonary infections in approximately 80–90% of human clinical cases with *A. flavus*, *A. terreus*, and *A. niger* causing the remainder. It has been estimated that 300,000 people in the world have IPA with between 1 and 10% of immunocompromised patients at risk. Although azole-mediated therapies with voriconazole have a 15% percent greater success rate with IPA than non-azole treatments [7], failure rates up to 50% in some studies are still quite high with the first line therapy [5, 8, 9]. Also worrisome is the developing resistance of *Aspergillus* spp. including *A. fumigatus* to azoles such as voriconazole in some areas of the world, particularly since the survival rate is substantially lower in patients with resistant strains. In tertiary centers in England that specialize in treating *Aspergillus* infections, resistance of the *A. fumigatus* strains now approaches 10% [10], resulting in longer hospital stays, higher costs, and poorer outcomes in these patients. Consequently, improvement in antifungal therapy by developing novel agents remains a priority.

This study focuses on development of branched histidine-lysine peptides (bHKP) to treat diseases caused by *Candida* and *Aspergillus* species. bHKP have previously shown activity against an array of fungi including *Candida*, *Aspergillus*, and *Cryptococcus* infections [11, 12]. Because these organisms are unable to synthesize branched peptides, cationic microbial peptides derived from living organisms are linear, which may limit their antimicrobial activity. In contrast, synthetic bHKP with varied numbers of branches can easily be made with a peptide synthesizer. Importantly, the pattern and the ratio of histidines to lysines may also be readily varied with bHKP which can modify its antifungal activity and safety profile. Specific histidine and lysine patterns enhance the efficacy of bHKP as antifungal agents. In addition, the activity of bHKP, perhaps due to its greater cationic charge, is minimally affected by isotonic media compared to linear histidine-rich peptides or histatin-5 [11, 13]. Although bHKP have similarities with histatin-5 in their relative selective antifungal activity, it is not clear if they have a similar antimicrobial mechanism [14, 15]. Recently, we demonstrated that addition of a targeting ligand to bHKP significantly increased antifungal activity [13]. Here, we modified a 4-branched bHKP, H3K4b, with D-amino acids and form silver adducts with H3K4b (and analogues) to enhance antifungal efficacy.

2. Materials and Methods

2.1 Fungi

The following fungi were obtained from the American Type Culture Collection (ATCC; Manassas, VA): *Candida albicans* (ATCC 10231), *Aspergillus fumigatus* (ATCC 13073), *A. flavus* (MYA3631) and *Aspergillus niger* (ATCC 1015); These fungi were grown in yeast maltose medium (Becton Dickinson, Sparks, MD).

2.2. Synthesis of peptides

The biopolymer core facility at the University of Maryland synthesized the bHKP in Table 1 on a Ranin Voyager solid-phase synthesizer (PTI, Tucson, AZ) as previously described. If peptide purity was less than 95%, then peptides were further purified on a high-performance liquid chromatography column by using a Dynamax 21-4 X250 mm C-18 reversed-phase preparative column. Further analyses of the polymers were performed with a Voyager MALDI-TOF mass spectroscopy (Applied Biosystems, Foster City, CA). The molecular weight of H3K4b and analogues is 9,555.

2.3. Antifungal activity of bHKP

Antifungal efficiency of bHKP was determined by measuring the growth of fungal cells in 96 well microtiter plates reader. Fungal cells were diluted at 5×10^4 cells/ml in RPMI 1640-MOPS (20 mM, pH-7.0) medium [16]; 90 μ l of cell or spore suspensions were added to a 96 well culture plate. bHKP (10 μ l) were added to each well, mixed well, and then incubated at room temperature for 24h. After incubation, the turbidity of the suspension, a measure of fungal growth was measured at 590 nm with a microplate reader. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the peptide or peptide/silver combination that resulted in at least 95% inhibition from untreated control, whereas MIC₅₀ was defined as the peptide concentration which resulted in 50% inhibition of control. MIC and all additions of bHKP and silver include their salts, which are approximately 40% of their weight (bHKP-trifluoroacetate~ 42%; AgNO₃~36.5%).

2.4. WST-1 Cell viability Assay

As previously described [13], metabolic activity was also assayed using the WST-1 assay of treated and control fungal cells.

2.5. Reactive Oxygen measurements

The intracellular level of reactive oxygen species (ROS) was measured by hydroxyphenyl fluorescein (HPF) (Invitrogen, Eugene OR). The non-fluorescent HPF becomes fluorescent in the presence ROS. After exposure to antimicrobial agents for 24 h, HPF (10 μ M) was added for 1h and images were captured with a Nikon TE2000 fluorescence microscope (Ex-485; Em-534).

2.6 Silver adduct formation with bHKP

The interaction between H3K4b (or analogue) and Ag⁺ was examined as previously described [17]. With final concentration of the bHKP held constant (0.5 μ g/ μ l), different amounts of AgNO₃ (Sigma-Aldrich, St. Louis, MO) were added (0.000625 to 0.005 μ g/ml); this corresponded to ratios of HK: Ag of 1:800 to 1:100. After incubation in MOPS buffer (total volume 10 μ l, 20 mM, pH-7.0) for 30 min, the UV spectrum was obtained with a Nano Spectrophotometer (Shimadzu, Kyoto, JP). The peak interaction between silver and histidine (imidazole) was measured at an OD of 232 nm.

2.7 Varying the Addition Order of bHKP and AgNO₃

Three conditions were done to determine the antifungal efficacy of adding H3K4b and silver to medium containing *A. fumigatus*: 1) mixing H3K4b (5 µg) with AgNO₃ (concentrations ranging from .005 to 0.05 µg) together for 30 min prior to adding this adduct to the medium; 2) add AgNO₃ and then 30 min later, add H3K4b; 3) add H3K4b and then 30 min later, add AgNO₃.

2.8 Size of H3k4b: Ag Conjugates

Preparation of H3k4b-silver conjugates were prepared in MOPS buffer (pH-7.0) as described above. Thirty min after adding AgNO₃ to H3k4b at varying ratios, the size of H3k4b: Ag conjugates were determined with dynamic light scattering (DLS) (Malvern).

2.9 Cytotoxicity assay

Cells (NHDF, MDA-MB-435, CHOs, and BAECs) were cultured in 24-well plates with DMEM medium. After cells reached 70% confluence, increasing amounts of bHKP (range, 0 to 120 µg/ml) were added to triplicate wells. After incubation for 24 h, cells were stained with crystal violet (1%) and their number was determined.

3. Results

3.1 Antifungal Efficacy of bHKP

Because H3K4b has greater antifungal activity than H2K4b [13] and has reduced toxicity when exposed to mammalian cells, H3K4b was selected to evaluate D-lysine and/or D-histidine substitutions in the branches (e.g., H3k4b). By incorporating D-amino acids into the branches, we investigated whether enhanced antifungal activity of H3K4b would occur. Interestingly, replacement of L-lysines with its D-stereoisomer (designated H3k4b) improved antifungal activity toward *A. fumigatus* and *C. albicans* (Table 2). The MIC with modified H3k4b and H3K4b were 45 µg/ml (~4.5 µM) and 90 µg/ml (~9 µM), respectively, against *A. fumigatus*. When L-histidines were replaced with D-histidines in the branches (i.e., h3K4b), the antifungal activity was also greater than that of H3K4b. In contrast, replacement of both histidine and lysine with D-stereoisomers (h3k4b) resulted in a decrease in activity compared to the selectively modified H3K4b (i.e., h3K4b, H3k4b). Against *C. albicans*, differences among the analogues were more evident with MIC₅₀. The MIC₅₀ of H3k4b and h3K4b were reduced by more than 3-fold compared to H3K4b and h3k4b.

Although bHKPs demonstrated less activity against *C. albicans* than *A. fumigatus*, the ranking of peptides by their antifungal efficacy was similar. Because the mitochondria of fungi have been a proposed target of antimicrobial agents including histatin-5 [18], free oxygen radicals generated by H3k4b was investigated. With increased concentrations of H3k4b, greater generation of free oxygen radicals occurred (Fig. S1). Further corroborating the growth inhibitory results, the metabolic activity of *C. albicans* was significantly reduced by H3k4b compared to H3K4b and untreated groups at 24 h (Fig. S2).

3.2. The combination of silver and HK improved antifungal efficacy

Because transitional metals, including Ag^+ , form coordination bonds with imidazole, which is the side group of histidine, we were interested in whether the combination of bHKP and Ag^+ would form adducts that enhance antifungal activity. We initially used the most effective bHKP (H3k4b and h3K4b) to combine with the Ag^+ . The activity of either bHKP or AgNO_3 against *C. albicans* and *A. fumigatus* was markedly improved by the combination (Table 3). For *C. albicans*, the silver adduct of bHKP (H3k4b and h3K4b) reduced the MIC compared to the peptide and silver alone by 4- and 5-fold, respectively.

For *A. fumigatus*, the combination of HK and silver showed particular promise (Table 3). Although the combination of h3K4b or H3k4b with AgNO_3 was quite effective, the unmodified H3K4b and AgNO_3 combination was surprisingly more effective and showed the greatest synergistic effect (Table 3, S1). The combination of H3K4b and AgNO_3 reduced the MIC compared to the peptide or AgNO_3 alone by 30- and 26-fold, respectively (Table 2, 3). The marked fractional reduction of the H3K4b- AgNO_3 combination compared to the product of AgNO_3 and peptide inhibition further underscored the antifungal synergy of the combination (Table S1) [19]. Consistent with these results, significant increases in free oxygen radicals were produced with the H3k4b and silver combination (Fig. 1).

Not all bHKP or lysine-containing peptides showed enhanced and/or synergistic activity with the addition of the silver ion. For both *A. fumigatus* and *C. albicans*, the all D- h3k4b analogue silver combination was significantly less effective than either the h3K4b- or H3k4b-silver combination. Moreover, the branched K4b and AgNO_3 combination demonstrated reduced synergy compared to histidine-containing peptides (Table 3, S1).

3.3. Evidence that an adduct is formed and is necessary for synergistic activity

Based on literature, histidine interacts with Ag^+ through coordination bonds with the imidazole side group. With increases in absorption at 232 nm, spectrophotometry provides evidence that an adduct is formed between histidines in peptides and Ag^+ [17]. We found that UV absorption progressively increased as the amount of AgNO_3 was added to bHKP (Fig. 2), indicating an adduct was formed. In addition, flame spectroscopy supported that an adduct had formed by measuring the pre- and post-washings level of silver in the presence or absence of the peptide (data not shown). With DLS, there was no evidence that aggregates (vs. adducts) were formed with ratios used for these antifungal studies. Nevertheless, aggregates to some degree were formed when ratios of bHKP: AgNO_3 were 5:1, indicating intermolecular interactions between bHKP- Ag -bHKP species. Specifically, whereas the size of the bHKP: silver conjugate at a ratio 100:1 (wt:wt) was no different than the peptide alone (0.68 nm), the size of bHKP: silver conjugate at a ratio of 5:1 was about 9-fold greater (6.3 nm).

Further evidence that an adduct was formed came from the order of addition studies. When AgNO_3 was added to the medium containing *A. fumigatus* 30 min prior to addition of H3K4b, the antifungal activity was markedly reduced by 90% compared to co-administering H3K4b and AgNO_3 (Fig. 3). Similarly, addition of bHKP prior to AgNO_3 reduced

antifungal efficacy compared to coadministration by 83% at the most inhibitory dosage. This suggested that an adduct was necessary for full activity of the H3K4b and AgNO₃.

3.4. Low cellular toxicity of HK and/or silver adduct

We did not observe any significant effects on growth of Chinese Ovary cells, fibroblasts, or endothelial cells with H3K4b or H3k4b when concentrations up to 120 µg/ml (~12 µM) were present in the medium (Table S2). With other peptides or with adducts, minimal cellular toxicity was observed at the highest concentrations.

4. Discussion

Antimicrobial synergy of silver with antibiotics was recently reported by Morones-Ramirez et al [20]. By increasing reactive oxygen species, silver potentiated the antimicrobial activity of gentamycin and vancomycin *in vitro* and *in vivo*. However, since most antibiotics including vancomycin or gentamycin do not form adducts with silver, pathogenic organisms may be exposed to marked variations in the amounts of silver or the antimicrobial agent. When the target cell is the same for both AgNO₃ and the antimicrobial agent, their ability to form an adduct may increase the probability that the pathogenic organism will be exposed to both active agents, thereby augmenting the synergy of the drugs.

Perhaps more important, targeting the H3K4b:Ag conjugate to the pathogenic organism is certainly possible whereas targeting Ag⁺ administered separately from the peptide would be problematic. Recently bHKP with cRGD attached to the N-terminal ends of each branch showed enhanced antifungal activity against *C. albicans* [13]. αvβ3 integrins, in which cRGD ligands bind tightly, are increased on the surface of the *C. albicans* as well on the spores of aspergillus species. Other cyclic ligands, such as -CGGRLGPFC-, selectively bind to the conidida and hyphae of Aspergillus spp. [21, 22], and show promise for targeting antimicrobial peptides. We think that coordination of Ag⁺ with histidines of the H3K4b is unlikely to affect targeting since the ligands are conjugated to the N-terminal ends of the branches. Thus, we anticipate that enhancement of antifungal activity will occur when adducts are formed between Ag⁺ and H3K4b modified with ligands.

Although silver adducts with histidine-rich peptides have not been previously described to enhance antifungal activity, the interaction between transitional metals including silver and histidine (imidazole) groups has been well-characterized [23]. Since Ag⁺ binds with high affinity through coordination bonds with 2 histidines [24, 25], H3K4b with 48 histidines in its 4 branches can readily accommodate one or two Ag⁺, thereby reducing the likelihood that peptide complexes will be formed, particularly when the Ag⁺ concentration is limited. At ratios used to determine the MIC, there was no evidence of aggregate formation by dynamic light scattering. With the possible exception of topical use, peptide-silver adducts that do not form aggregates or nanoparticles [26] may be preferable for improved penetration of infected tissues.

We did not anticipate that the D-lysine H3k4b would have greater antifungal activity than the all D-h3k4b. Although one might surmise that breakdown of bHKP would lead to less activity, this would not readily explain why H3k4b had greater activity than the all D-h3k4b

analogue. Our data is consistent with the hypothesis that partial breakdown of bHKP is necessary for optimal antifungal activity. However, the reduced antifungal activity of H3K4b against *C. albicans* compared to several analogues is likely due to its marked proteolysis by this organism.

Building on these likely differences in stability and antifungal activity, stability of bHKPs has an even more striking role in the synergistic activity between peptides and AgNO₃. Although H3K4b targeting *A. fumigatus* had less inhibitory effect than other analogues, the H3K4b-silver combination showed greater efficacy than other bHKP-silver analogues. Moreover, the synergistic antifungal activities between several H3K4b analogues (H3K4b, h3K4b, and H3k4b) and AgNO₃ were increased compared to K4b and h3k4b. We think the variation of synergy between silver and these peptides provides insight into their antifungal activity.

First, partial breakdown of bHKP appeared necessary for optimal synergy to occur between these peptide and silver. The reduced synergy with the all-D form (h3k4b), which is likely more stable enzymatically compared to the all L- or partial D-isomeric forms of H3K4b (i.e., H3K4b, H3k4b, h3K4b), suggests that partial breakdown of the peptide has an important role in synergy with silver. Moreover, H3K4b, which is likely most vulnerable to enzymatic hydrolysis, had the greatest synergistic activity with silver against *A. fumigatus* compared to the other peptides. This supports that partial degradation of branched peptides is a condition necessary for greater synergistic activity. Partial breakdown of bHKP would likely preserve activity of the peptide while enabling release of silver from the adduct. In contrast to *A. fumigatus*, *C. albicans* showed less sensitivity to the H3K4b:Ag adduct, which may be due to marked proteolysis of the H3K4b by this organism. Thus, a balance between synergy and peptide degradation appears necessary to achieve maximal antifungal activity.

Second, the data are consistent with formation of an adduct being necessary to obtain optimal synergy. Although H3k4b and Ag⁺ form adducts as indicated by our studies, this does not provide biological evidence that an adduct is necessary for optimal synergy. However, evidence that an adduct is essential for their full antimicrobial activity is suggested by the lack of synergy between silver and K4b, a peptide that does not readily form coordination bonds with silver. Evidence that an adduct was necessary for optimal synergy also comes from experiments of adding peptides and silver sequentially to fungi. The sequential addition of bHKP and silver (or the reverse) to the medium showed decreased antifungal activity compared to co-administration of bHKP and the Ag⁺, a condition favorable for adduct formation.

The sequential addition of peptide and silver experiments also suggests that internalization of the adduct was necessary for full activity. If the more accessible cell membranes were the primary site of action of the adduct or one of its components, one would expect that the sequential addition of Ag⁺ and then bHKP (or the reverse) might have similar or even greater activity than the adduct. That the antifungal activity was markedly reduced with sequential addition supports that the target of the bHKP and Ag⁺ was intracellular. The high intracellular uptake of fluorescein-labeled H3K4b is also consistent with an intracellular target for the adduct [13]. Thus, several lines of evidence indicate that bHKP-Ag adducts are

transported intracellularly into the pathogenic fungus and that subsequent limited breakdown of the peptidesilver adduct leads to optimal antifungal synergy.

In summary, selective replacement of L-histidines or -lysines with D-isomers in the branches of H3K4b increased the antifungal activity of these analogues. Moreover, when AgNO₃ was added to the H3K4b or an analogue, evidence supports that an adduct was formed and that partial degradation of the adduct improved antifungal activity. Although the H3K4b-Ag adduct was most effective against *A. fumigatus*, the H3K4b-Ag adduct exhibited a broader range of antifungal activity against *C. albicans* and *A. fumigatus*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

bHKP	branched histidine-lysine peptides
ROS	reactive oxygen species
HPF	hydroxyphenyl fluorescein
DLS	dynamic light scattering
MIC	minimal inhibitory concentration

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Fig. 1. Combination therapy augments intracellular levels of reactive oxygen species
HPF was used to indicate the level of reactive oxygen species in *A. fumigatus*, after the combined treatment of Ag^+ and H3K4b (A) Untreated; (B) AgNO_3 , 0.03 $\mu\text{g/ml}$; (C) H3K4b (3.0 $\mu\text{g/ml}$); (D) AgNO_3 (0.03 $\mu\text{g/ml}$)+ H3K4b (3.0 $\mu\text{g/ml}$). The bar represents 50 μm .

Fig. 2. Interaction of bHKP and silver ion

With UV spectroscopy, interaction of Ag^+ with histidines of bHKP was determined at 232 nm. Ratio of H3K4b: AgNO_3 varied from 800/1 to 100/1. See “Material and Methods” for further details.

Fig. 3. Adduct increases efficacy

The antifungal efficacy of co-administration of H3K4b (30 μ g) and AgNO₃ (0.003–0.030 μ g) was compared to that of sequential additions of H3K4b and 30 min later the AgNO₃ (or AgNO₃ and then H3K4b) to medium with *A. fumigatus*. *, $P < 0.05$, **, $P < 0.01$, ANOVA, H3K4b-Ag adduct vs. sequential additions of H3K4b/AgNO₃ or AgNO₃/H3K4b.

Table 1

Peptide Sequences of H3K4b and analogues

bHK Peptides	Sequence	D and/or L a.a in branch	Number of a.a. in branch
H3K4b	[K-H-H-H-K-H-H-H-K-H-H-H-K] <u>4</u> Lys	L-his, L-lys	17-mer
H3 <u>k</u> 4b	[k-H-H-H-k-H-H-H-k-H-H-H-k] <u>4</u> Lys	L-his, D-lys	17-mer
<u>h</u> 3K4b	[K-h-h-h-K-h-h-h-K-h-h-h-K] <u>4</u> Lys	D-his, L-lys	17-mer
<u>h</u> 3 <u>k</u> 4b	[k-h-h-h-k-h-h-h-k-h-h-h-k] <u>4</u> Lys	D-his, D-lys	17-mer
K4b	[K-K-K-K-K-K-K-K-K-K-K-K-K-K-K-K] <u>4</u> Lys	L-lys	17-mer

H3K4b, a bHKP with 4-branches and a predominant repeating pattern of –KHHHK– in its branches; H3**k**4b, analogue of H3K4b in which L-lysines in branches are replaced with D-lysines; **h**3K4b, analogue of H3K4b in which L-histidines are replaced by D-histidines; **h**3**k**4b, H3K4b analogue in which both L-histidines and L-lysines are replaced by D-stereoisomers. K4b, 4 branched peptide in which branches contain lysines. Branches of peptides emanate from a three L-Lys core (in bold).

Table 2MIC and MIC50 ($\mu\text{g/ml}$) of bHK Peptides

Fungi	<i>C. albicans</i>	<i>A. fumigatus</i>	<i>A. flavus</i>	<i>A. niger</i>
Peptide	MIC (MIC50)			
h3K4b	120 (9)	45 (3)	45 (3)	0.3
H3k4b	120 (9)	45 (3)	45 (3)	0.6
h3k4b	>150 (30)	150 (12)	-----	1.5
H3K4b	>150 (30)	90 (6)	----	0.6
K4b	>150 (90)	>150 (15)	----	----

MIC (MIC50 in parenthesis) were determined by adding bHKPs (range 0 to 150 $\mu\text{g/ml}$) to the microplate cultures. After 24 h, the optical density was measured at 590 nm by a microplate reader.

Table 3MIC of bHKP and AgNO₃ Combination

Fungi bHKP\Ag	<i>C. albicans</i>	<i>A. fumigatus</i>	<i>A. flavus</i>
Ag ⁺	1.5	0.4	0.4
H3k4b\Ag	30\0.3	3\0.03	3.0\0.03
h3K4b\Ag	30\0.3	3\0.03	----
h3k4b\Ag	45\0.45	12\0.12	----
H3K4b\Ag	60\0.6	3\0.015	----
K4B\Ag	>60\0.6	>12\0.12	----

MIC (μg/ml) were determined by adding peptides (0.3 to 12 μg/ml, *Aspergillus*; 10 to 60 μg/ml, for *Candida*) in combination with AgNO₃ at a weight ratio (bHKP\AgNO₃) of 100:1 (~molar ratio, 1:1) or 200:1 to microplate cultures. After 24 h, the optical density was measured at 590 nm. Peptide and AgNO₃ concentrations precede and follow the backslash, respectively. Optimal combination of bHKP and AgNO₃ is in bold.