Biochemical and Biophysical Research Communications 477 (2016) 957–962

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Silver adducts of four-branched histidine rich peptides exhibit synergistic antifungal activity

Qixin Leng [a], Martin C. Woodle [b], Yijia Liu [b], A. James Mixson [a, *]

[a] Department of Pathology, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[b] Aparna Biosciences Corp, Rockville, MD 20852, USA

ARTICLE INFO

Article history:
Received 27 June 2016
Accepted 2 July 2016
Available online 4 July 2016

Keywords:
Antimicrobial peptides
Candida
Aspergillus
Silver
Therapy

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 *Candida 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 *Aspergillus 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* 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 *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus 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

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

* Corresponding author.
E-mail address: Jmixson@som.umaryland.edu (A.J. Mixson).

http://dx.doi.org/10.1016/j.bbrc.2016.07.008
0006-291X/

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 9555.

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 μl of cell or spore suspensions were added to a 96 well culture plate. bHKP (10 μl) were added to each well, mixed well, and then incubated at room temperature for 24 h. 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%; $AgNO_3$~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 μM) was added for 1 h 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 μg/μl), different amounts of $AgNO_3$ (Sigma-Aldrich, St. Louis, MO) were added (0.000625–0.005 μg/ml); this corresponded to ratios of HK: Ag of 1:800 to 1:100. After incubation in MOPS buffer (total volume 10 μl, 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 $AgNO_3$

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 $AgNO_3$ (concentrations ranging from 0.005 to 0.05 μg) together for 30 min prior to adding this adduct to the medium; 2) add $AgNO_3$ and then 30 min later, add H3K4b; 3) add H3K4b and then 30 min later, add $AgNO_3$.

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-H-H-H-K]$_4$**Lys**	L-his, L-lys	17-mer
H3k4b	[k-H-H-H-k-H-H-H-k-H-H-H-k-H-H-H-k]$_4$**Lys**	L-his, D-lys	17-mer
h3K4b	[K-h-h-h-K-h-h-h-K-h-h-h-K-h-h-h-K]$_4$**Lys**	D-his, L-lys	17-mer
h3k4b	[k-h-h-h-k-h-h-h-k-h-h-h-k-h-h-h-k]$_4$**Lys**	D-his, D-lys	17-mer
K4b	[K-K-K-K-K-K-K-K-K-K-K-K-K-K-K-K]$_4$**Lys**	L-lys	17-mer

H3K4b, a bHKP with 4-branches and a predominant repeating pattern of –KHHHK- in its branches; H3k4b, analogue of H3K4b in which L-lysines in branches are replaced with D-lysines; h3K4b, analogue of H3K4b in which L-histidines are replaced by D-histidines; h3k4b, 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).