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Regular Articles

Effective inhibition of Cbf-14 against *Cryptococcus neoformans* infection in mice and its related anti-inflammatory activity

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ABSTRACT

Cbf-14 (RLLRKFFRKLKKSV), a designed peptide derived from cathelicidin family AMP, has proven to be potent against drug-resistant bacteria. In the present study, we investigated the anti-cryptococcal activity of Cbf-14 *in vitro* and in a pulmonary infection mouse model. Sensitivity test indicated that Cbf-14 possessed effective antifungal activity against *Cryptococcus neoformans* with an MIC of 4–16 μg/ml, and killing experiments showed that fungicidal activity was achieved after only 4 h treatment with Cbf-14 at 4× MIC concentrations *in vitro*. Meanwhile, Cbf-14 was effective at prolonging the survival of infected mice when compared with controls, and significantly inhibited the secretion of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, suggesting its anti-inflammatory activity against fungal infections. As a positively charged peptide, Cbf-14 was proven to neutralize the negative zeta potential of the fungal cell surface, disrupt the capsule polysaccharide of fungi, and further damage cell membrane integrity. These results were confirmed by flow cytometry analysis of the fluorescence intensity after PI staining, while cell membrane damage could be clearly observed by transmission electron microscopy after Cbf-14 (4× MIC) treatment for 1 h. In addition, Cbf-14 increased the IL-10 levels in cultured RAW 264.7 cells, which were stimulated by *C. neoformans* infection. The obtained data demonstrated that Cbf-14 could rapidly kill *C. neoformans* cells *in vitro*, effectively inhibit *C. neoformans* induced-infection in mice, and inhibit inflammation *in vitro / vivo*. Therefore, Cbf-14 could potentially be used for the treatment of fungal infections clinically.

1. Introduction

Despite many advances in antifungal therapeutics, fungal infections continue to affect many individuals and thus place a significant burden on the healthcare system and the economy (Brown et al., 2012). Meningitis and meningoencephalitis, which are caused by *C. neoformans*, have a high morbidity and mortality rate in immunocompromised individuals, especially in patients with AIDS (Park et al., 2009). Such a poor outcome is partly due to the extremely limited number of clinically available antifungal agents. Currently, the clinical treatment for cryptococcosis depends on amphotericin B (AmB) and azole antifungals. As first line therapy for cryptococcal meningitis treatment, current guidelines recommend 2 weeks of amphotericin B intravenously in combination with flucytosine (Perfect et al., 2010). However, this regimen remains widely unavailable in most parts of the world due to the high cost of flucytosine (Rajasingham et al., 2012). Azole drugs are not as effective as amphotericin B, which is not well-tolerated long term, but the inevitable toxicity of amphotericin B to kidneys and adrenal glands greatly limits its clinical applications (Laniado-Laborin and Cabrales-Vargas, 2009). While, azole drugs have serious side effects through interacting with some cytochrome P_{450} proteins in human body and easily occur cross-resistance problems during overlong term administration (Carmona and Limper, 2017). Therefore, the investigation of new drugs with potent anti-cryptococcal activity is urgently needed (Choi et al., 2010).

Antimicrobial peptides (AMPs) represent attractive candidates for the generation of new anti-mycotics. AMPs are a large family of small peptides (< 10 kDa) that are produced by almost all unicellular and multicellular organisms, and constitute a major part of the innate immune response against microbial pathogens (Cederlund et al., 2011). Due to their preferential interactions with prokaryotic and fungal membranes, these peptides provide a rapid and broad-spectrum response against both Gram-negative and Gram-positive bacteria, as well as against fungi (Lehrer and Ganz, 2002). Additionally, recent studies

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have found that antimicrobial peptides also inhibit the release of inflammatory cytokines and thus induce an anti-inflammatory effect (Sun and Shang, 2015).

Cbf-14 is a 14 amino-acid peptide designed independently in our laboratory with a distinguishing structure mutated from a cathelicidin family AMP from the vertebrate *Bungarus fasciatus* (banded krait). In previous research, Cbf-14 (RLLRKFFRKLKKSV) (Ma et al., 2016) has been proven to be potent to inhibit drug-resistant bacteria. However, it is still unknown whether Cbf-14 possesses antifungal activity, especially when considering the differences in the structure between bacteria and fungi.

In the present study, we elucidated the antifungal activities of Cbf-14 against *C. neoformans in vitro* as well as its *in vivo* effectiveness on a fungal-infection mouse model. We also investigated the mechanism of its action on membranes and performed its safety evaluation, which demonstrated the therapeutic potential of the peptide for the treatment of fungal infections.

2. Materials and methods

2.1. Materials

The peptide Cbf-14 (RLLRKFFRKLKKSV) and FITC-Cbf-14 was synthesized by GL Biochem Corporation (Shanghai, China). Propidium iodide was purchased from eBioscience (SanDiego, CA, USA).

2.2. Fungal strains

Cryptococcus neoformans ATCC4906 was obtained from the American Type Culture Collection. The clinical isolates, *C. neoformans* 1, 2, 3, 4 and 5 strains were obtained and identified by the Dermatology Hospital of Chinese Academy of Medical Sciences (Nanjing, China) and kept at −70 °C until analysis.

2.3. Animals

ICR male mice, purchased from the Laboratory Animal Center of Yangzhou University (Yangzhou, China), were provided with standard rodent chow and water. All animal procedures in this study were performed in strict accordance with protocols approved by the Ethics Committee of China Pharmaceutical University.

2.4. Antifungal activity assay in vitro

The minimal inhibitory concentration (MIC) of the peptide Cbf-14 was measured using a standard serial dilution method. The minimal fungicidal concentration (MFC) was tested using the method which has been employed from the National Committee for Clinical Laboratory Standards (Ji et al., 2014). Media with Cbf-14 concentrations ranging from 256 to 0.5 μg/ml were incubated with log-phase fungal cells (1×10^5 CFU/ml) for MIC determination. The 1 × MIC–16 × MIC samples were then absorbed into the sterile plate, and were cultured at 30 °C for 48 h.

2.5. Time-killing kinetics of Cbf-14 against C. neoformans cells

The capability of Cbf-14 in killing *C. neoformans* cells was assessed by using a standard microbiological method with a few modifications (Tavanti et al., 2011; Wang et al., 2014). Briefly, mid-log phase *C. neoformans* cells were diluted to 1×10^5 CFU/ml and incubated with Cbf-14 at 4 × MIC concentration for 0, 0.5, 1, 2, 4, 8, 12 and 24 h. At each time point, samples were diluted to three different dilutions and cultured for fungal count. Each measurement was performed in duplicate.

2.6. The construction of C. neoformans-infection mouse model

Encapsulated *C. neoformans* clinical strains were obtained from the Dermatology Hospital of Chinese Academy of Medical Sciences (Nanjing, China). *C. neoformans* cells were diluted to 2.0×10^8 cells/ml with sterile PBS. To induce immunosuppressed mice, all animals were given daily doses of cyclophosphamide (100 mg/kg, *i.p.*) which began three days before the challenge. On the day of inoculation (Day 0), 50 μl prepared cell suspension was slowly inoculated into the left nostril of each animal(Sabiiti et al., 2012; Zhai et al., 2013), which was anesthetized with diethyl ether inhalation, for the construction of the pulmonary infection mouse model (Kwon-Chung et al., 2014).

2.7. Therapy regimens

Mice were fully recovered and divided randomly into six groups: Control (no infection), Model (0.9% saline), Cbf-14 (2.5, 5, 10 mg/kg/d), and miconazole nitrate (100 mg/kg/d). Five animals per group were used for the fungal burden assay and histopathological analysis; ten animals per group were used for the survival study and weight change observation. Once-daily therapy was initiated immediately after the infection and given for five consecutive days. Mice were injected through the lateral tail vein for Cbf-14 or 0.9% saline administration and gavaged for miconazole nitrate administration (Perfect et al., 1996).

2.8. Survival and CFU assay

After infection, mice were treated in the presence or absence of Cbf-14 and were checked twice daily to record the survival rate. Simultaneously, during the total 22 days of treatment, animals were weighed every 2 days to investigate the change in bodyweights. The mice lungs and brains were aseptically collected and weighed to determine the tissues' fungal burden of pre-infection, post-infection and the fifth day after 24 h of the last administration. For each mouse, the whole lung and brain were homogenized in 9-fold cold PBS with a homogenizer (FLUKO, Shanghai, China) for the CFU assay and subsequent cytokine analysis. The fungal burden is given as the $\log_{10}$ CFU per gram of tissues (Zhai and Lin, 2013).

2.9. Histopathological analysis

To estimate lung damage, the sections were routinely stained with hematoxylin and eosin (H&E). To visualize the distribution of the invasive *C. neoformans*, the sections were stained with periodic acid-Schiff (PAS) reagent (Papareddy et al., 2013).

2.10. Microscale thermophoresis (MST)

Glucuronoxylomannan (GXM), a major polysaccharide component in the capsule of *C. neoformans,* was isolated as described previously (Cherniak et al., 1991; Villena et al., 2008). The interactions between FITC-Cbf-14 and GXM were analyzed by Microscale Thermophoresis (MST) experiments using a Monolith NT.115 instrument (Nanotemper Technologies, Munich, Germany) (Rogez-Florent et al., 2017).

2.11. Size distribution measurements

The size distribution of GXM (1 mg/ml) in the absence or presence of Cbf-14 (32 ug/ml) were determined at 25 °C by Zeta Potential & Particle Size Analyser as previously described (Domingues et al., 2008).

2.12. Flow cytometry assay of Cbf-14 affinity to the C. neoformans cell membrane

The membrane integrity was determined by FACScan analysis *via*