International Journal of Antimicrobial Agents 41 (2013) 250–254

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Evaluation of the anticryptococcal activity of the antibiotic polymyxin B in vitro and in vivo

Bing Zhai, Xiaorong Lin*

Department of Biology, 3258 Texas A&M University, College Station, TX 77843-3258, USA

ARTICLE INFO

Article history:
Received 13 August 2012
Accepted 2 November 2012

Keywords:
Cryptococcosis
Cryptococcus neoformans
Polymyxin B
Fluconazole
Capsule

ABSTRACT

Polymyxin B (PMB), a cationic lipid oligopeptide used to treat Gram-negative bacterial infections, was previously identified to possess broad-spectrum antifungal activity and to work synergistically with azole antifungals in vitro. Here we evaluated the efficacy of PMB against *Cryptococcus neoformans* in vitro and in vivo and explored the mechanism of the hypersensitivity of this fungus to this compound. Using comparative time-course assays, PMB was found to kill both proliferative and quiescent cryptococcal cells in vitro. Presence of the polysaccharide capsule, a characteristic feature of *Cryptococcus*, significantly enhances the susceptibility of this fungus to the fungicidal activity of PMB. Furthermore, PMB is able to reduce the tissue fungal burden both in intravenous and inhalation models of murine cryptococcosis at a level comparable with the commonly used antifungal fluconazole. These findings suggest that PMB could provide an additional option for treatment against systemic cryptococcosis.

1. Introduction

Invasive fungal infections have emerged worldwide as a serious threat to public health owing to the increasing population of immunocompromised patients such as individuals infected by human immunodeficiency virus (HIV) and recipients of cancer chemotherapy or tissue transplantation [1,2]. For example, systemic cryptococcosis, predominantly caused by the fungal pathogen *Cryptococcus neoformans*, is responsible for ca. 25–30% of deaths of acquired immune deficiency syndrome (AIDS) patients (over 0.6 million) each year [3]. Cryptococcosis in heart and small bowel transplant recipients has a mortality rate of 50% [4]. Such poor outcome is partly due to the extremely limited number of clinically available antifungal agents. Currently, the clinical treatment for cryptococcosis relies on amphotericin B, which was discovered over 60 years ago [5], and azole antifungals [commonly fluconazole (FLC)] [6]. The fungistatic nature of azoles renders it necessary to apply azoles as long-term maintenance treatment, which could lead to the emergence of resistant fungal strains [7]. Therefore, it is important to investigate new compounds to enrich the repertoire of antifungal agents.

Through a screen of the Johns Hopkins Clinical Compound Library (Johns Hopkins School of Public Health, Baltimore, MD), we found that the antibiotic polymyxin B (PMB) possesses antifungal activity. It can act either alone or in combination with azoles against various species of fungal pathogens [8]. PMB is a cationic lipid oligopeptide that is mostly used to treat infections caused by multidrug-resistant Gram-negative bacteria [9,10]. This positively charged molecule has a neutralising effect on the negatively charged bacterial outer membrane largely due to the presence of lipopolysaccharide. This electrostatic interaction is the critical initial step [11]. Therefore, PMB perturbs the integrity of the bacterial membrane, eventually resulting in cell lysis [12]. Several other cationic peptide antibiotics, such as polymyxin E and omiganan, also display antifungal activity [13,14]. It has been proposed that these compounds also disrupt the integrity of plasma and/or vacuolar membranes in eukaryotic fungi and cause cell lysis. Compared with *Candida* or *Aspergillus* spp., the fungus *Cryptococcus* is more susceptible to PMB [8]. Among fungi, the presence of a polysaccharide capsule is a unique feature in *Cryptococcus*. The capsule is an important virulence factor as it protects *Cryptococcus* cells from many environmental and host-relevant stresses, such as phagocytosis [15]. Production of the capsule is greatly induced under host-relevant conditions (high temperature, high CO_2 concentration, and neutral or alkaline pH). Because this polysaccharide layer primarily contributes to the high negative charge of cryptococcal cells [16], we suspect that the presence of the capsule may contribute to the hypersusceptibility of *Cryptococcus* to PMB.

Clinically achievable serum concentrations of PMB are ca. 6.25–50 mg/L [17], which are below the in vitro inhibitory concentrations against most fungal pathogens but fall within the range of those of *Cryptococcus* isolates [8,14]. Therefore, we decided to evaluate the in vivo efficacy of PMB in two murine models of cryptococcosis. Naturally, hosts inhale cryptococcal cells from the

* Corresponding author. Tel.: +1 979 845 7274; fax: +1 979 845 2891.
E-mail address: xlin@bio.tamu.edu (X. Lin).

0924-8579/$ – see front matter © 2012 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.
http://dx.doi.org/10.1016/j.ijantimicag.2012.11.006

environment. The fungal cells are either cleared in the lungs or establish latent infections [18]. When the immune system of the host is compromised (e.g. HIV infection), cryptococcal cells can activate, disseminate from the lungs and eventually cause fatal systemic cryptococcosis [18]. In the inhalation model, animals are inoculated intranasally with *Cryptococcus* cells, which is analogous to the natural route of infection. These animals primarily develop pulmonary infections. In the intravenous (i.v.) model, animals are infected intravenously with fungal cells to represent disseminated cryptococcosis. Although the poor penetration of PMB to the central nervous system may limit its effect on brain infections, our data on the tissue fungal burden of the lungs and kidney suggest a modest efficacy of this compound against systemic cryptococcal infections.

2. Materials and methods

2.1. Strains and media

The *Cryptococcus* strains used in this study include the serotype A reference strain H99, the capsule mutant strain *cap59*Δ derived in the H99 background [19] and the serotype D strain XL280 [20]. Strains were maintained on yeast–peptone–dextrose (YPD) medium (Difco, Houston, TX). Time-course assays were performed using phosphate-buffered saline (PBS) or RPMI-1640 medium buffered with 4-morpholinepropanesulfonic acid (MOPS) (BioWhittaker™; Lonza Inc., Allendale, NJ).

2.2. Compounds and animals

For in vitro studies, polymyxin B sulfate was dissolved in water at a stock concentration of 20 mg/mL and FLC was dissolved in water at a stock concentration of 2 mg/mL; both of the drugs were further diluted with PBS buffer or RPMI medium to the indicated working concentrations. For animal studies, PMB was dissolved in 0.9% saline at a stock concentration of 20 mg/mL and was diluted with 0.9% saline for injection. FLC was dissolved in 0.9% saline at 4 mg/mL. Female A/J mice (6–8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME).

2.3. In vitro time-course assays of effect of the capsule on drug efficacy

Time-course assays were employed to evaluate the effect of the polysaccharide capsule on drug efficacy. To induce capsule production, wild-type H99 cells were cultured on RPMI agar medium at 37 °C in 5% CO_2. To suppress capsule production, H99 cells were cultured on YPD agar with addition of 1 M NaCl at 30 °C in ambient air. After 3 days of incubation, the cells grown under each condition were collected and washed twice in PBS buffer. The cells were then suspended in PBS buffer to obtain a cell density of 1500–2000 cells/mL. The cell suspensions obtained from these two different growth conditions were then treated with PMB (8 mg/L). A no-drug treatment was used as the positive control. At different time points (0, 1, 2, 4, 6 and 8 h post inoculation), aliquots of cell suspensions were spread onto drug-free solid YPD medium to determine the number of viable cells by colony counting.

2.4. In vitro study of the effect of the capsule on drug efficacy using an acapsular mutant

To quantify the MIC_{100} of PMB against the wild-type H99 strain and the *cap59*Δ mutant, fungal cells of the two strains were cultured in liquid YPD medium overnight (30 °C, 250 rpm). Cells were then collected by centrifugation and washed twice in PBS buffer. Cells were inoculated into RPMI medium to reach a density of 1500–2000 cells/mL. The stock PMB solution was directly diluted in RPMI medium to reach drug concentrations of 0, 2, 4, 6, 8, 10, 12, 14, 16 and 20 μg/mL. Wells that contained no *Cryptococcus* cells were included as negative controls. The MIC_{100} of PMB was defined as the lowest drug concentration that resulted in a 100% decrease in absorbance at an optical density of 600 nm compared with that of the control in drug-free medium.

2.5. In vivo murine models of cryptococcosis

Animal models of systemic cryptococcosis were induced by two infectious routes, namely i.v. and intranasal infection. In each infection model, mice were assigned to four treatment groups: control (0.9% saline); FLC; PMB; and the drug combination. Five animals per group were used for the fungal burden assay and ten animals per group were used for the survival study. All of the drugs were administered intraperitoneally. For the i.v. infection, each mouse was challenged with 1.0×10^4 H99 cells at Day 0. Drug treatment with FLC (10 mg/kg/day), PMB (2.5 mg/kg/day) or the drug combination started on Day 1 and lasted for 5 consecutive days. Mice were given drugs once every 24 h. Mice were then sacrificed on Day 6 and their kidneys were harvested for determination of tissue fungal burden. For the intranasal infection, animals were first sedated with ketamine and xylazine and then 1.0×10^5 H99 cells suspended in 50 μL of saline were slowly inoculated into the left nostril of sedated animals. In the survival study, drug treatment with FLC (16 mg/kg/day), PMB (2.5 mg/kg/day) or the drug combination was initiated at Day 2 and lasted for 5 consecutive days. In the fungal burden study, drug treatment with FLC (4 mg/kg/day), PMB (2.5 mg/kg/day) or the drug combination was initiated at Day 10 and lasted for 3 consecutive days. Mice were sacrificed 24 h after the last dose of treatment and their lungs were harvested. The kidneys or lungs were homogenised in 2 mL of cold PBS buffer using an IKA® Ultra-Turrax® T-18 Homogenizer (IKA Works, Wilmington, NC) with the same setting for each type of organ. Tissue suspensions were serially diluted (10×), plated onto YPD agar and incubated at 30 °C for 2 days such that the colonies became visible in order to calculate CFUs. Animals were weighed daily and were monitored twice a day for disease progression and potential severe side effects, including weight loss, gait changes, laboured breaths or fur ruffling. Animal experiments were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University (College Station, TX) (animal protocol permit no. 2011-22).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) tests in the fungal burden studies were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). A P-value of <0.05 was considered significant.

3. Results

3.1. Polymyxin B is fungicidal against both proliferative and non-proliferative cryptococcal cells in vitro

Our previous study indicated that PMB possesses antifungal activity and can dramatically enhance the potency of FLC against many fungal pathogens in vitro [8]. These assays were performed with fungal cells undergoing active proliferation. To examine whether PMB is fungicidal both against proliferative and non-proliferative cryptococcal cells, a comparative time-course assay was performed of cell viability of H99 and XL280 strains in vitro treated with PMB, FLC or the drug combination. Fungal cells in RPMI