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Research Article

ANTIFUNGAL AND ANTIBIOFILM ACTIVITY OF 2-BROMO-*N*-PHENYLCETAMIDE AGAINST *CRYPTOCOCCUS NEOFORMANS*

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ABSTRACT

Objective: This study aimed to evaluate the antifungal and antibiofilm of 2-bromo-*N*-phenylacetamide (A1Br) against *Cryptococcus neoformans in vitro*.

Methods: The compound was characterized by infrared and ^{1}H-nuclear magnetic resonance spectrometry data. Minimum inhibitory concentration (MIC) was determined using the plate dilution method and biofilm formation inhibition of the crystal violet assay.

Results: A1Br inhibited the growth of *C. neoformans* with MIC of 0.25 and 0.5 µg/ml and has good antibiofilm activity MIC ×4, with ≥80% inhibition growth.

Conclusions: Thus, A1Br shows potential antifungal and antibiofilm agents for the treatment of infections caused by *C. neoformans*, as well as the eradication of cryptococcal colonization of medical prosthetic devices.

Keywords: Antifungal, Biofilm, 2-Bromo-*N*-phenylacetamide, *Cryptococcus neoformans, In vitro*.

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INTRODUCTION

Cryptococcosis is a predominantly opportunistic fungal infection caused by yeasts of *Cryptococcus* genus, belonging to *Cryptococcus gattii* and *Cryptococcus neoformans* complexes. This disease is associated with one of the main causes of mortality in immunocompromised individuals, predominantly HIV/AIDS [1-3].

Infection occurs through inhalation of the yeast's infectious forms, the basidiospores, usually in soil contaminated with decomposing bird and vegetable excrement. As a result, the manifestations usually occur in the pulmonary and cerebral form, often causing the most severe state of the disease, meningoencephalitis [4].

The pathogenicity of species of the genus *Cryptococcus* is facilitated by the expression of several virulence factors that play an important role in colonization, adhesion, invasion, dissemination, and evasion of the fungus from the defense mechanisms of the host's immune system [5]. Among these, factors are the ability to adhere to host cells and/or medical devices, production of exoenzymes, and biofilm formation [5-8].

Biofilms are defined as communities of highly organized microorganisms involved in a matrix polymeric, which are composed mainly of DNA, proteins, and polysaccharides [9].

Biofilm formation is an important virulence factor and is associated with increased antifungal resistance as it makes it harder for antifungal agents to penetrate the extracellular matrix, in addition to protecting the fungal cells from the host's immune response and environmental stress [10,11]. The eradication of biofilms *in vivo* generally requires concentrations of antimicrobials that are generally toxic to the host. In *C. neoformans*, biofilms are more resistive than planktonic cells to amphotericin B, in addition to being generally resistant to fluconazole and voriconazole [12]. To reverse this scenario, several studies have been carried out to screen for new compounds that have antifungal activity.

The amide group is widely found in natural and synthetic products and important biological properties are described. Among the amides, some studies verified the efficacy of acetanilide derivatives, related to a wide range of biological activities, such as anti-inflammatory [13,14], analgesic [13,15], antibacterial [13,16,17], antiparasitic [13,18,19], and antifungal [13,16].

Considering, *C. neoformans* biofilm infections are increasingly recognized as a clinical problem. The discovery of new chemical entities effective in combating fungal resistance and with low toxicity is necessary and urgent. This work aims to investigate antifungal activity and the ability of 2-bromo-*N*-phenylacetamide (A1Br) to interfere in the inhibition of biofilm formation in *C. neoformans* isolates.

METHODS

Test substance and antifungal drugs

Aniline, bromoacetyl bromide, potassium carbonate (K_2CO_3), chloroform, and ethanol were purchased from Sigma-Aldrich (São Paulo, Brazil). The substance A1Br was prepared in a 50 mL flask containing aniline (1.86 g; 0.020 moles) and K_2CO_3 (3.31 g;0.024 moles) solubilized in 20 ml of $CHCl_3$ at a temperature of 0°C, 2-bromoacetyl bromine (4.84 g; 0.024 moles) is added slowly. The bath ice is removed and the reaction is stirred for 20 h at room temperature environment. The reaction mixture is accompanied by thin-layer chromatographic (1:1 hexane/ethyl acetate). At the end of the reaction, the reaction mixture was subjected to extraction. The phrase organic is washed with water (3 ml × 25 ml), dried over anhydrous sodium sulfate, and then concentrated at reduced pressure, providing the precipitate

that was recrystallized in an ethanol/water (8:2) mixture providing 80% yield. The A1Br compound was characterized by ^{1}H-nuclear magnetic resonance spectrometry data and infrared (IR) and the data corroborated with Xie *et al*. [20].

Voriconazole and amphotericin B (Sigma-Aldrich®, São Paulo, SP, Brazil) were dissolved in 5% dimethyl sulfoxide and 2% Tween 80 (Sigma-Aldrich®, São Paulo, Brazil), and using sterile distilled water.

Culture, microorganisms, and inoculum preparation

Strains of *C. neoformans* clinical (LM-22 and LM-525) and standard origin INCQS 40221 (National Institute of Quality Control in Health) were used.

The strains were kept on Sabouraud dextrose agar (SDA) at 4°C until the inoculum was prepared. The preparation of the inoculum was carried out in inclined sterile tubes containing SDA and incubated at 35° ± 2°C for 72 h. After the incubation period, a fungal suspension was prepared and adjusted on the 0.5 McFarland scale.

To test the inhibition of biofilm formation, the culture medium Roswell Park Memorial Institute (RPMI)-1640-L-glutamine (without sodium bicarbonate) was used (Sigma-Aldrich®, São Paulo, SP, Brazil).

Determination of minimum inhibitory concentration (MIC)

MIC was performed using the broth microdilution technique [21-23]. Briefly, 100 µl of RPMI-1640 doubly concentrated were distributed

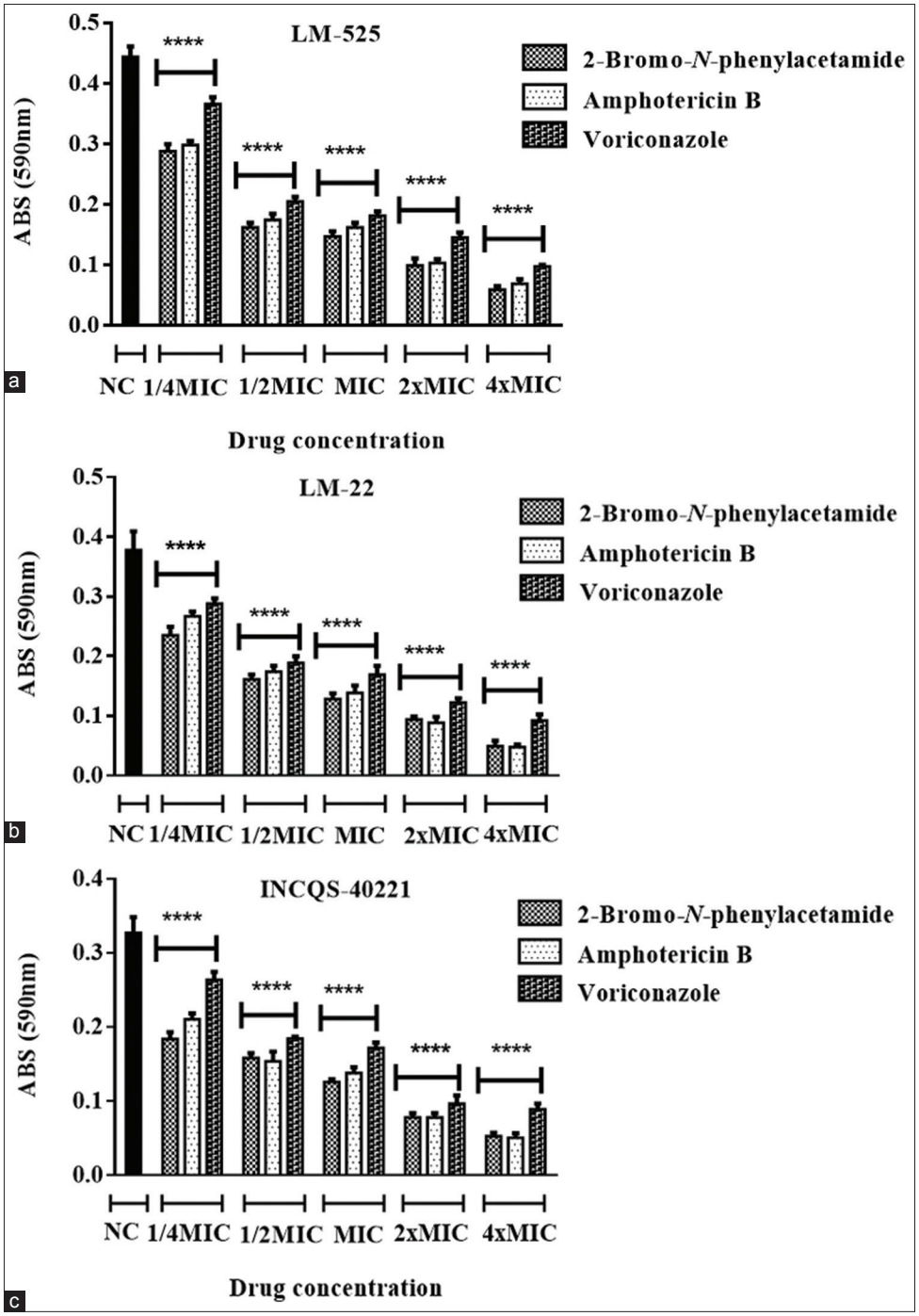

Fig. 1: (a-c) The effect of inhibiting biofilm formation 2-bromo-*N*-phenylacetamide, amphotericin B, and voriconazole in concentrations ranging from 1/4 to ×4 minimum inhibitory concentration

to the microdilution plate wells. Then, 100 µl of A1Br (also doubly concentrated) was dispensed in serial dilution in initial concentrations of 1024–0.007 µg/ml. Then, 10 µl aliquots of the inoculum suspension of the strain were added to the wells. At the same time, sterility control and negative control were performed. The plates were incubated at 35±2°C for 72 h. The results were analyzed observing the turbidity of the medium that is indicative of the growth of the microorganism. MIC is defined as the lowest concentration that produces visible inhibition of yeast growth compared to controls. They were expressed as the arithmetic means of the MIC obtained, performed in triplicate.

A1Br antifungal activity was interpreted and considered active or inactive, according to the following criteria: Strong activity: 50–500 µg/ml; moderate activity: 600–1500 µg/ml; and weak or inactive activity: >above 1500 µg/ml [24].

Biofilm formation inhibition assay

In the biofilm inhibition assay, 10 µl of *C. neoformans* fungal cell suspension was dispensed on microtiter plates with 100 µl of RPMI medium containing different concentrations of A1Br (¼ MIC, ½ MIC, ½ MIC, MIC × 2, and MIC × 4) on each item. After 72 h of incubation at 35 ± 2°C, the contents were discarded from the wells and washed with sterile distilled water. At room temperature, drying occurred and 140 µl of 1% violet crystal solution (NEWPROV®) was added over 40 min. After discarding the dye and the excess is removed with distilled water. After drying, 140 µL of absolute ethanol (RIOQUÍMICA®) was added for 15 min, measured in a Multiskan GO microplate reader at 595 nm. The negative control was performed. The analyzes were performed in triplicate. This test was performed according to the methodology described by Balasubramanian *et al*. [25], with modifications.

Statistical analysis

Statistical analyzes were performed to determine significant differences between the treated and untreated groups by unidirectional analysis of variance (ANOVA) followed by the Bonferroni post-test. The results were plotted using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). The statistical difference was established at values of $p<0.05$, represented by asterisks in the figures.

RESULTS AND DISCUSSION

The results obtained for MICs of A1Br against *C. neoformans* were 0.25 and 0.5 µg/ml (Table 1). Given this result, we can classify the substance as an optimal activity, since, according to Sartoratto *et al*. [24], the MIC results will be considered as an excellent activity if the value is 50–500 µg/ml; moderate: 600–1500 µg/ml; and weak or inactive activity when the value is above 1500 µg/ml.

To date, this is the first study in the literature on the antifungal activity A1Br. However, there are studies with chloroacetamide derivatives, in which Katke *et al*. [26] evaluated the antimicrobial activity of these derivatives using the disk diffusion method and considered strong activity. Machado *et al*. [27] verified the antifungal activity of 11 chloroacetamide derivatives using MIC, only three were effective against dermatophytes (MIC: 3.12–50 µg/ml) and *Candida* species (MIC: 25–50 µg/ml).

The formation of biofilm represents a major problem in therapy, due to the concentration required to eliminate it, it may exceed permitted doses of the antimicrobial [28,29].

The ability of A1Br to inhibit the biofilm formation of *C. neoformans in vitro* was evaluated. The A1Br molecule showed inhibition capacity at all concentrations tested. In the treated and control groups, significant differences were observed (Fig. 1a-c). It was observed in the graph that A1Br presents good antibiofilm activity [30] MIC ×4, which corresponds to ≥80% inhibition of biofilm growth to the dose-dependent concentration, the higher the concentration, the better inhibition capacity.

After 1/4 MIC, there was a reduction of more than 40% in the biofilm formation by *C. neoformans*. This study demonstrates sub-1/4 MIC of A1Br had a better ability to inhibit biofilm formation than voriconazole ($p\leq0.0001$) (Fig. 1a-c). However, this reduced action on the biofilm may be associated with protection of the exopolymeric matrix, composed mainly of glucuronoxylomannan in the biofilm cells, forming a physical barrier that makes it difficult for voriconazole to reach the cell [12,31]. Besides, other mechanisms such as increased gene expression and/or efflux pump activity in biofilm-forming cells, compared to planktonic antifungals, may be associated with this reduction.

Amphotericin B and A1Br inhibited over 80% of all biofilms of *C. neoformans* strains at concentrations ≥0.5 and 1 µg/ml, respectively (Fig. 2). In the study by Martinez and Casadevall [12], it was observed

Table 1: MIC of the 2-bromo-*N*-phenylacetamide against of *Cryptococcus neoformans*

Strains	Minimal inhibitory concentration 2-bromo-*N*-phenylacetamide (mg/ml)	Control*
INCQS 40221	0.5	+
LM-22	0.5	+
LM-525	0.25	+

MIC: Minimum inhibitory concentration, *Control of microbial growth

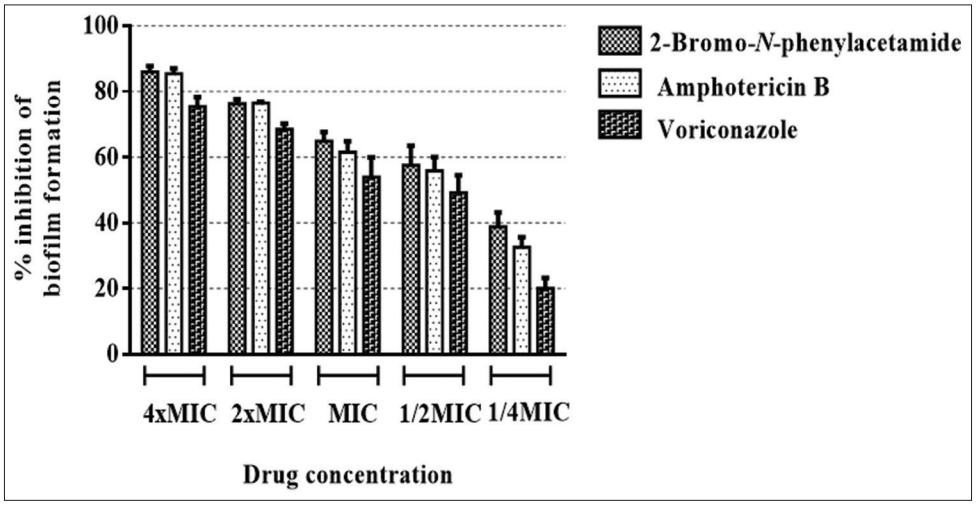

Fig. 2: The percentage of inhibition of the biofilm formation of the strains was examined at final concentrations of 2-bromo-*N*-phenylacetamide ranging from 1/4 to ×4 minimum inhibitory concentration

that the survival of biofilms was reduced by approximately 50% after treatment with 4 µg/ml of amphotericin B and 80% of biofilm *C. neoformans* were inhibited after treatment with 8 µg/ml of amphotericin B. Therefore, A1Br has shown very good antifungal and antibiofilm activity against biofilm-forming *C. neoformans.*

CONCLUSIONS

The search for the medicinal chemical that exhibits antifungal and antibiofilm activities has been of interest since the rise of antimicrobial resistance. In this study, the antifungal and antibiofilm action of compound 2-bromo-*N*-phenylacetamide can make it a promising new antifungal agent in the treatment of cryptococcosis. Additional studies are needed for the elucidation of antibiofilm activity in the capsule and adhesion mechanism.

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AUTHORS' CONTRIBUTIONS

Thamara Melo, Laisa Cordeiro, Helivaldo Souza, and Sávio Ferreira contributed to carrying out experiments, analyzing data, and preparing the manuscript. Dra. Edeltrudes Lima, Dr. Abrahão Oliveira, and Petrônio Athayde-Filho contributed to designing the experiment and put forwarded valuable suggestions.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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