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ARTICLE

In vitro interactions of amphotericin B and non-antifungal compounds against opportunistic human pathogen *Cryptococcus neoformans*

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ABSTRACT The incidence of opportunistic human pathogen *Cryptococcus neoformans* caused infections have been higher during the last few decades along with the increasing number of susceptible individuals around the world. Recommended treatment of cryptococcal meningoencephalitis is a combined therapy with amphotericin B deoxycholate and flucytosine. Despite of the efficiency of this drug combination, the mortality rate of the disease is high due to the limited accessibility and the high cost of these antifungals in the most severely affected areas. The broad-spectrum activity of non-antifungal drugs and their potential to enhance the efficiency of conventional antifungal agents have been recognised previously. In this study, the *in vitro* activity of amantadine, valproic acid, trifluoperazine and chlorpromazine was tested against five *C. neoformans* strains individually and in combination with amphotericin B. All the four compounds exerted slight antifungal activity against the studied *C. neoformans* strains. Their combination with amphotericin B revealed additive and synergistic interactions.
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Introduction

The encapsulated basidiomycetous yeast, *Cryptococcus neoformans* is distributed world-wide mainly in association with bird excrement (Srikanta et al. 2014). The species is an opportunistic human pathogen and can cause serious disease primarily in immunocompromised individuals, i.e. HIV-positive patients, patients with organ transplants undergoing immunosuppressive therapy and cancer patients going through chemotherapy; making them vulnerable to fungal infection. The infection of immunocompetent hosts is rare. The disease caused by *C. neoformans* is called cryptococcosis. The infection starts with the inhalation of the airborne basidiospores or dried cells (Köhler et al. 2015). The spores germinate in the lungs, thereafter the cells disseminated by the blood stream can reach and colonize the central nervous system establishing meningoencephalitis. Cryptococcosis affects about 1 million people in the world - most of them are HIV-infected - and causes the death of more than 600 000 patients per year (Warkentien and Crum-Cianfloan 2010). The majority of the cases are registered in certain parts of Africa and Asia where the incidence of HIV-infection is higher (Sloan and Parris 2014).

A combined antifungal therapy involving amphotericin B deoxycholate and flucytosine is recommended for the treatment of cryptococcal meningoencephalitis (Day et al. 2013). However, flucytosine is an unregistered drug in most parts of Asia and Africa and its cost is high because of the limited number of manufacturers and these factors make the administration of this drug near impossible (Loyse et al. 2013).

Many non-antifungal pharmaceuticals have an antifungal side effect. Some of them can act alone while others can enhance the activity of antifungal agents when used together (Afeltra and Verwe 2003; Judd and Martin 2010; Nyilasi et al. 2010). Among the non-antifungals, the activity of phenothiazines like trifluoperazine and chlorpromazine, have been studied in detail. However, the antifungal activity of amantadine and valproic acid were only recognised recently against opportunistic human pathogenic fungal species (Wood and Nugent 1985; Eilam et al. 1987; Homa et al. 2015; Chaillat et al. 2017). Amantadine is an ion channel blocker used to treat Parkinson's disease (Blanpied et al. 2005). The anti-epileptic drug, valproic acid inhibits the action of histone deacetylases (HDACs) and induces the degradation of HDAC2 (Göttlicher 2004). The antipsychotic drugs chlorpromazine and trifluoperazine exert their antifungal activity via arresting the cell cycle and destroying the cell membrane integrity in the susceptible species (Eilam et al. 1987). All

Table 1. List of the tested strains

Species	Strain number
Cryptococcus neoformans	IFM 5844
Cryptococcus neoformans	IFO 410
Cryptococcus neoformans	SZMC 26851
Cryptococcus neoformans	SZMC 26852
Cryptococcus neoformans	IFM 48637

IFM: Culture Collection of the Research Centre for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan
IFO: Institute for Fermentation, Osaka, Japan
SZMC: Szeged Microbiological Collection

these drugs can penetrate across the blood brain barrier and can act in the central nervous system.

The aim of this study was to test the *in vitro* anti-*Cryptococcus* activity of amantadine, chlorpromazine, trifluoperazine and valproic acid against five *C. neoformans* strains, and to evaluate their interaction with amphotericin B.

Materials and methods

Yeast strains and growth conditions

The *C. neoformans* strains used in the present study are listed in Table 1. The strains were cultivated on Yeast Peptone Dextrose medium (YPD, 0.5% yeast extract, 1% peptone, 1% dextrose, 2% agar) at 30 °C for 48 hours and were kept at 4 °C until use.

The experiments were carried out with actively growing cells; therefore, a single colony was transferred to 2 mL sterile YPD medium and incubated at 30 °C for overnight. Cells were then harvested by centrifugation at 10000 rpm for 5 minutes in Heraeus Pico 17 centrifuge (Thermo Scientific, Waltham, MA, US) and washed twice with sterile distilled water, finally they were suspended in RPMI 1640 medium (Sigma-Aldrich, Germany).

Non-antifungal compounds

Amantadine hydrochloride, chlorpromazine hydrochloride, trifluoperazine hydrochloride, valproic acid sodium salt (Sigma-Aldrich, Germany) and amphotericin B (AppliChem, Darmstadt, Germany) were provided by the manufacturers as standard powder. The non-antifungal compounds were dissolved in 96% ethanol while amphotericin B in dimethyl sulfoxide (DMSO) to prepare stock solutions (10 mg/mL and 1 mg/mL, respectively) which was stored at -20 °C until used. Further dilutions were performed in RPMI 1640 medium.

Antifungal activity assays

Determination of Minimal Inhibitory Concentration (MIC)

The antifungal effect of the drugs was determined by broth micro-dilution assay in 96-well flat bottom microplate. Fifty µl serially twofold diluted compounds were added to 50 µl of standardized cell suspension (8 x 10^4 cell/mL in RPMI 1640 medium). The final concentration of the amphotericin B was ranged from 0.156 to 5.0 µg/mL, and those of amantadine, chlorpromazine, trifluoperazine and valproic acid from 7.81 to 500 µg/mL. The control samples contained 50 µl cell suspension and 50 µl RPMI 1640 medium. Solvent control was used to check the effect of the ethanol and DMSO on the growth rate of the strains.

The plates were incubated at 30 °C for 48 h. At the end of the incubation, the optical density of the samples was detected at 620 nm in SPECTROstar Nano plate reader (BMG LabTech, Offenburg, Germany). The experiments were carried out at least three times always in triplicates. The MIC was defined as the concentration of the compound caused total inhibition of cell growth.

Interaction between amphotericin B and the non-antifungal compounds

The *in vitro* interaction of the compounds and amphotericin B was determined by standard checkerboard titration method. The amphotericin B was tested in a concentration range from 0.156 to 2.5 µg/mL while the concentration ranges of all the other compounds varied from 7.81 to 125 µg/mL. The cell concentration in each well was 4 x 10^4 cell/mL. After the incubation for 48 h at 30 °C, the optical density of the cultures was detected at 620 nm

Table 2. Antifungal activity of the compounds

Species	Minimal inhibitory concentrations (µg/mL)				
	amphotericin B	amantadine	chlorpromazine	trifluoperazine	valproic acid
Cr. neoformans IFM 5844	0.625	>500	125	62.5	>500
Cr. neoformans IFO 410	0.625	>500	62.5	62.5	>500
Cr. neoformans SZMC 26851	0.625	>500	125	62.5	>500
Cr. neoformans SZMC 26852	0.625	>500	125	62.5	>500
Cr. neoformans IFM 48637	0.625	>500	125	62.5	>500

Table 3. Interaction ratios between amphotericin B and amantadine, chlorpromazine, trifluoperazine and valproic acid after 48-h incubation at 30 °C

	Drugs	Strains				
		IFM 5844	IFO 410	SZMC 26851	SZMC 26852	IFM 48637
AMB +	amantadine	0.93 ADD	1.04 ADD	0.82 ADD	0.70 ADD	0.92 ADD
	chlorpromazine	0.94 ADD	1.04 ADD	0.85 ADD	0.88 ADD	0.97 ADD
	trifluoperazine	1.29 ADD	1.02 ADD	1.51 SYN	1.29 ADD	1.09 ADD
	valproic acid	1.07 ADD	0.86 ADD	1.88 SYN	1.12 ADD	1.55 SYN

AMB: amphotericin B; ADD: additive interaction; SYN: synergistic interaction

in SPECTROstar Nano plate reader (BMG LabTech, Offenburg, Germany). The MIC was determined for each compound alone and in combinations. The experiments were carried out at least three times always in triplicates.

Data analysis

For calculation of the inhibition rates, the absorbencies of the untreated control cultures were assumed to be 100% growth in each case. Expected efficacy of each combination was determined by the Abbott formula: $I_e = X + Y - (XY/100)$, where I_e is the expected percent inhibition for a given interaction, and X and Y are the percent growth inhibited by the compounds when used alone. The nature of interaction of these antifungal compounds was determined by the interaction ratios (IRs), which were computed as $IR = I_o/I_e$ (I_o, observed percent inhibition). IRs between 0.5 and 1.5 represent additive interactions, ratios of >1.5 represent synergistic interaction, and ratios of <0.5 represent antagonistic interactions.

Results

Antifungal activity of the tested drugs

The antifungal activities are summarised in Table 2. All the examined strains were slightly susceptible to the drugs. Among the non-antifungal compounds, the MIC of trifluoperazine proved the lowest: 62.5 μg/mL. Chlorpromazine showed the same MIC (62.5 μg/mL) for *C. neoformans* IFO 410 strain, all the other strains were less susceptible, as the MIC was 125 μg/mL in that case. The MIC of amantadine and valproic acid could not be established as it was out of the applied concentration range. The MIC of amphotericin B was 0.625 μg/mL for each strain.

Interaction between amphotericin B and the non-antifungal compounds

Positive interactions were detected between the amphotericin B and each compound. All the tested drugs augment the effectiveness of the amphotericin B against *C. neoformans* strains as additive and synergistic interactions occurred between them (Table 3). Using *C. neoformans* SZMC 26851 as susceptible strain synergism was detected combining amphotericin B either with valproic acid or trifluoperazine. Valproic acid and amphotericin B combination showed synergistic interaction against IFM 48637 strain too. All the other combinations demonstrated additive interactions between amphotericin B and the drugs against the tested strains.

Discussion

Cryptococcosis is a world-wide infectious disease associated mainly with immunodeficient hosts. The disease most commonly manifests as cryptococcal meningitis. However, pulmonary and primary cutaneous cryptococcosis also exist (Sloan and Parris 2014). As other invasive fungal infections, cryptococcosis is associated with high morbidity and mortality rate. Particularly the treatment of cryptococcal meningoencephalities affecting the central nervous system is difficult because amphotericin B having significant role in the treatment penetrates poorly across the blood brain barrier due to its relatively high molecular weight (Nau et al. 2010). Additional problem is the low accessibility of the other recommended drug, flucytosine (Loyse et al. 2013).

The in vitro broad-spectrum activity of non-antifungal compounds against human pathogenic fungi was published earlier (Judd and Martin 2009). Testing the activity of phenothiazines such as chlorpromazine and trifluoperazine against medically important yeasts such as *C. neoformans* proved that it is one of the most susceptible species (Eilam et al. 1987). Although, the anti-*Cryptococcus* activity of these compounds has been established earlier, their interaction with amphotericin B was not investigated. In this present study, the in vitro action of chlorpromazine, trifluoperazine, valproic acid and amantadine individually and in combination with amphotericin B were studied. The results showed that all the examined compounds possess antifungal activ-

ity as they slightly reduced the growth of *C. neoformans* strains when applied alone. Trifluoperazine was the most efficient drug as it had the lowest MIC against all the five strains involved in this study. The drugs and amphotericin B established additive or synergistic interactions as in combination with amphotericin B they achieved more effective growth inhibition than being used alone. Amphotericin B in combination with the studied drugs attained more efficient growth reduction in lower concentrations than used alone.

The positive interaction between the drugs and amphotericin B can be explained by the ability of amphotericin B to bind to the ergosterol and forming pores in the fungal cell membrane (Gallis et al. 1990). Non-antifungal agents could enter the cells via these pores and could exert their activity within the fungal cell. Amantadine, chlorpromazine, trifluoperazine and valproic acid accumulates in the central nervous system and there is potential to apply them in combination with amphotericin B in the treatment of *Cryptococcus*-caused meningoencephalitis.

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