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BACTERIAL AND FUNGAL PATHOGENESIS - RESEARCH PAPER

In vitro combination with doxycycline plus antifungals against clinical Mucorales pathogens

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Abstract

Purpose Since systematic antifungals for mucormycosis showed variable MICs depending on strains, effective and safe antifungal therapy was still needed. This study is aimed to evaluate the in vitro activity of doxycycline combined with antifungal therapy against dominant Mucorales pathogens.

Methods Multidrug susceptibility testing was performed with doxycycline and antifungals, including itraconazole, posaconazole, and amphotericin, in 21 isolates of 8 dominant Mucorales pathogens.

Results The fractional inhibitory concentration index according to M38 showed one *Rhizopus arrhizus* isolate synergic ($\sum$FICI = 0.375) and other isolates in addition ($0.5 < \sum FICI < 4$).

Conclusions Doxycycline was found to have in vitro advantages in combined antifungal treatment over antifungals alone.

Keywords Mucormycosis · Doxycycline · Susceptibility test · *Mucor irregularis*

Introduction

More than 500 cases of mucormycosis were reported worldwide between 2013 and 2017 [1], while the COVID-19 pandemic has ushered in a surge of mucormycosis since 2019, especially in India [2]. Mucorales have been listed as WHO 'high priority' since October 2022 [3], and classified into 7 families including *Rhizopodaceae*, *Mucoraceae*, *Lichtheimiacede*, *Cunninghamellaceae*, *Thamnidiaceae*, *Saksenaeaceae*, and *Syncephalastraceae*. Mucorales pathogens varied causative proportions in order of *Rhizopus arrhizus*, *Lichtheimia corymbifera*, *Rhizopus microsporus*, *Cunninghamella bertholletiae*, *Apophysomyces elegans*, *Rhizomucor pusillus*, *Mucor* spp., exhibiting different disease manifestations and drug sensitivity [4].

For many years, amphotericin B (AMB), isavuconazole (ISA), and posaconazole (POS), as well as surgical debridement of necrotic tissue if necessary [5, 6], have become effective and recommended methods for mucormycosis [7]. In vitro, AMB exhibits optimal activity against almost all Mucorales pathogens [8, 9], while itraconazole (ITZ) has a strain-dependent variable Minimal Inhibitory

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Concentrations (MICs) [10], and POS has less activity than ISA but sensitive to *Rhizopus spp.* [11, 12]. Voriconazole and echinocandins have lower activity against Mucorales in vitro [13, 14]. However, in some developing countries, multiple options for the treatment of mucormycosis are unavailable [15], and the unbearable side effects limit its clinical application. Combination antifungal therapy should be considered to enhance efficacy, reduce medication dosage, and reduce adverse reactions in the treatment of mucormycosis.

Doxycycline (DOXY), a common broad-spectrum antimicrobial agent that has been tested in some clinics with rare and severe side effects [16, 17]. DOXY exerts antibacterial effects by binding to bacterial ribosomes to inhibit bacterial protein synthesis. However, the specific role it plays in fungi remains to be discussed. In the attempt to combine DOXY and fluconazole, 28% of *Candida glabrata* isolates showed synergy [18], while 50% of isolates of *Fusarium spp.* showed synergistic interactions when combine DOXY and AMB [19]. Therefore, we aim to evaluate the in vitro combination of DOXY *and* antifungal drugs, including ITZ, POS, and AMB, in eight dominant pathogenic Mucorales species. Here, we have found some evidence to support the optimization of the therapy regimen and direction of potential drug combinations.

Materials and methods

Fungal culture Twenty-one isolates were provided by the CAMS Collection Center of Pathogen Microorganisms-D (CAMS-CCPM-D) in Nanjing, China, including 2 *Rhizomucor pusillus*, 3 *Lichtheimia ramosa*, 3 *Syncephalastrum racemosum*, 3 *Rhizopus microsporus*, 1 *Cunninghamella homothallica*, 3 *Lichtheimia corymbifera*, 3 M.*irregularis*, 3 *Rhizopus arrhizus*, with 1 *Candida parapsilosis* (ATCC 22019) and 1 *Candida krusei* (ATCC 6258) set as quality-controlling strains.

Malt Extract Agar was employed as the culture medium plates (5% (w/v)) for growing Mucorales isolates at 30℃. After 96 h of culture, conidia were harvested, washed using PBS, and quantified to the concentration of 1×10^7 spores/ml. Quality-controlling (QC) strains were set with 1 *C. parapsilosis* (ATCC 22019) and 1 *C. krusei* (ATCC 6258), following Clinical and Laboratory Standards Institute (CLSI) guidelines, M38 [20]. QC strains were inoculated in Potato Dextrose Broth (PDB) at 30 °C for 8 h in a thermostatic shaker at 200 rpm, counted, and diluted as referred above.

Fungal isolates identification Genomic DNA samples cultured above were isolated from the culture colonies with EZNA™ Fungal DNA Miniprep Kits (Omega Bio-tek) according to the manufacturer's instructions, and quality control was subsequently carried out on the purified DNA samples. The PCR was performed using a Biometra TRIO Multi Block PCR Thermal Cycler. The PCR recipe was set as 5 μM of each primer (forward primer ITS1: 3′-TCCTCC GCTTATTGATATGC-5′, reverse primer ITS4: 5′-TCCTCC GCTTATTGATATGC-3′), for a final volume of 20 μl. The PCR temperature cycling used: initial denaturing at 95 °C for 5 min, followed by 45 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. PCR results were blasted against non-redundant (NR) in the NCBI database.

Drug susceptibility testing Twenty-one isolates were pre incubated in RPMI medium 1640 (Gibco 31,800–022) at 30 °C for 24 h. RPMI medium 1640 has been prepared with ddH_2O to pH = 7.4, sterilized and aseptically configured for double-dilution. All MIC tests were conducted in accordance with the current CLSI guidelines M38 [20]. A double dilution method was used to evaluate drug interactions as Fig. 1. The concentration ranges in combination assays were established based on the results of individual drug testing. In short, as shown in Fig. 1, a total of 10 double-diluted solutions of DOXY *(Aladdin D302150)* with 8 double-diluted solutions of either ITZ (Sigma I6657), POS (Flukar 32,103), or AMB (Sigma A9528) were prepared.

Statistical analysis MICs were read at 100% inhibition and analyzed using Microsoft Excel 2016. The fractional inhibitory concentrations index (FICI) was counted as below,

$$\begin{aligned}\textbf{FICI of antifungal agent} &= \text{MIC of the antifungal agent in combination} \\ &\div \text{MIC of the antifungal agent alone, and FICI of DOXY} \\ &= \text{MIC of DOXY in combination} \div \text{MIC of DOXY alone.}\end{aligned}$$

The total fractional inhibitory concentration ($\sum$FICI) for each isolate was calculated according to the formula:

$\sum$ **FICI** = FICI of antifungal agents + FICI of DOXY. ($\sum$FICI ≤ 0.5 means synergy, $\sum$FICI between 0.5 and 4 means addition, and $\sum$FICI ≥ 4 means antagonism [21].)

Results

The in vitro activity of the three single drugs and two combination methods were summarized in Table 1. The CLSI recommended 24-h MIC100 range of *Candida parapsilosis*

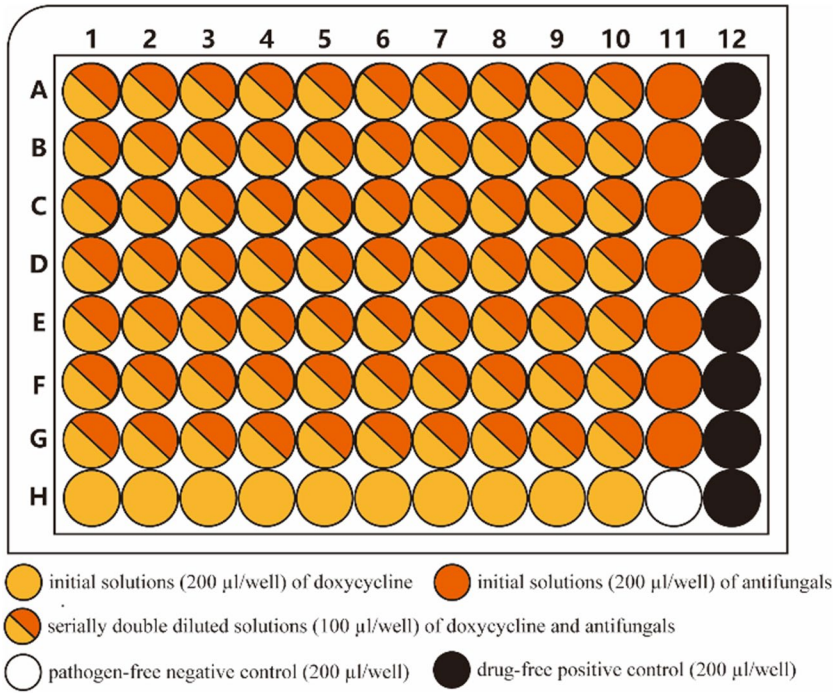

Fig. 1 Double-diluted solutions of DOXY, as well as ITZ, POS, or AMB were prepared and added as this diagram: The initial solutions (200 µl/well) of DOXY were dispensed into the bottom row of 96-well plates (yellow circles) and the serially double diluted ones (100 µl/well) were dispensed upward in turn (half yellow and half orange circles). Meanwhile, the initial solutions of antifungals (200 µl/well) were dispensed into the second rightmost column (orange circles) with the serially double diluted ones (100 µl/well) distributed to the left in turn (half yellow and half orange circles), leaving the rightmost column as a drug-free positive control (black circles) and one pathogen-free negative control (white circle)

ATCC 22019 was 0.06 to 0.5 µg/ml for ITZ, 0.03 to 0.25 µg/ml for POS, and 0.25 to 2 µg/ml for AMB [22], and trailing was not recognized as a problem in drug susceptibility testing for Mucorales. Therefore, all MICs were read at 100% inhibition. Quality control was performed at each testing event. As a result, the MIC100 range was 0.12 to 1 µg/ml, 0.06 to 0.5 µg/ml, and 0.5 to 2 µg/ml respectively for ITZ, POS, and AMB. All three replicates of QC strains had MIC100s within the CLSI acceptable ranges.

The MIC100s of ITZ ranged between 0.0625 µg/ml and 1 µg/ml for seven Mucorales species except for *Mucor irregularis*. The MIC100s of ITZ varied in *Rhizopus arrhizus* isolates. The MIC100s of POS showed similar conditions. AMB showed MIC100s = 0.5 µg/ml in *Rhizomucor pusillus* and *Syncephalastrum racemosum*, MIC100s = 1 µg/ml in *Lichtheimia ramosa* and *Lichtheimia corymbifera*, and MIC100s = 2 µg/ml in *Rhizopus microsporus*, *Cunninghamella homothallica*, *Mucor irregularis,* and *Rhizopus arrhizus*.

DOXY MIC100s for all species were > 64 µg/ml. A synergistic combination was only identified from the combination of DOXY and ITZ ($\sum$FICI = 0.375) for *Rhizopus arrhizus* strain B81f. The remaining combinations, including DOXY, POS, and AMB, showed additive interactions ($0.5 < \sum FICI < 4$).

Discussion

Due to the treatment options for mucormycosis being limited to antifungal drugss, as well as increased resistance and problematic side effects, we investigated the interactions between antifungal drugs and other drugs. DOXY and fluconazole showed a dose-dependent synergistic effect on clinical *Candida spp.* isolates and *Fusarium spp.*, converting fluconazole from fungistatic to fungicidal [18, 23]. Therefore, we performed in vitro susceptibility tests between DOXY and antifungal drugs including ITZ, POS, or AMB, to explore their combined effect.

Rhizomucor pusillus featured susceptibility to ITZ, POS, or AMB in antifungal monotherapy, while *Lichtheimia spp.* responded with similar activity to these three antifungals. Different *Rhizopus spp.* strains have distinct antifungal activity. *Syncephalastrum racemosum* was susceptible to AMB while *Cunninghamella homothallica* was tolerant to all antifungals tested. Against *Mucor spp.,* the MIC90s of ITZ and ISA were reported > 16 µg/ml, while POS varied from 0.125 µg/ml to 8 µg/ml [24]. *M.irregularis* POS MICs had been reported only in a few pieces of literature ranging from 0.25 to 2 µg/ml [25, 26]. AMB was active against all isolates of *M.irregularis*, while ITZ and POS had poor activity. However, AMB treatment for mucormycosis was often interrupted due to its drug side effects [27], which restricted its clinical application.

When DOXY and antifungal drugs were combined to treat Mucorales pathogens, $\sum$FICIs were between 0.5 µg/ml and 4 µg/ml, indicating a common additive effect. In our study, one *Rhizopus arrhizus* isolate showed synergy with DOXY and ITZ; the combination of DOXY and AMB made the additive effect more pronounced than other combinations. Although DOXY and AMB acted as additives in vitro, the interactions of DOXY

Table 1 The susceptibility results of DOXY combined with ITZ, POS, and AMB

Species	Strain No. (CAMS-CCPM-D)	DOXY	ITZ			POS			AMB		
			SINGLE DRUG	COMBINATION DRUG		SINGLE DRUG	COMBINATION DRUG		SINGLE DRUG	COMBINATION DRUG	
		MIC100	MIC100	MIC100 (ITZ/DOXY)	FICI<	MIC100	MIC100 (PCZ/DOXY)	FICI<	MIC100	MIC100 (AMB/DOXY)	FICI<
***Rhizomucor pusillus* (n=2)**	B63b	>64	0.0625	<0.0625/<0.125	1	0.5	0.25/32	1	0.5	0.0625/32	0.625
	B63c	>64	0.0625	<0.0625/<0.125	1	0.5	0.125/32	0.75	0.5	0.0625/32	0.625
***Lichtheimia ramosa* (n=3)**	B69f	>64	0.125	0.0625/64	1.5	1	0.5/32	1	1	0.0625/64	1.0625
	B69g	>64	0.125	0.0625/64	1.5	1	0.5/32	1	1	0.25/32	0.75
	B69h	>64	0.125	0.0625/64	1.5	1	0.5/32	1	1	0.25/32	0.75
***Syncephalastrum racemosum* (n=3)**	B78a	>64	0.25	0.125/32	1	1	0.5/32	1	1	0.125/32	0.625
	B78b	>64	0.25	0.125/64	1.5	1	0.5/32	1	0.5	0.25/32	1
	B78c	>64	0.25	0.125/32	1	2	0.5/64	1.25	0.5	0.125/32	0.75
***Rhizopus microsporus* (n=3)**	B96b	>64	0.25	0.125/32	1	1	0.5/64	1.5	1	0.25/32	0.75
	B96c	>64	0.25	0.125/64	1.5	1	0.5/64	1.5	2	0.125/64	1.0625
	B96e	>64	0.25	0.125/64	1.5	1	0.5/64	1.5	2	0.25/32	0.625
***Cunninghamella homothallica*(n=1)**	B59a	>64	0.25	0.125/32	1	1	0.5/64	1.5	2	1/64	1.5
***Lichtheimia corymbifera* (n=3)**	B63a	>64	0.125	0.0625/64	1.5	1	0.5/32	1	1	0.5/32	1
	B69a	>64	0.125	0.0625/64	1.5	2	1/32	1	0.5	0.25/64	1.5
	B69c	>64	0.125	0.0625/32	1	1	0.5/32	1	1	0.25/32	0.75
***Mucor irregularis* (n=3)**	B50a	>64	4	2/32	1	2	1/32	1	2	1/8	0.625
	B50m	>64	4	2/64	1.5	>4	4/16	1.25	1	0.0625/64	1.0625
***Rhizopus arrhizus* (n=3)**	B81a	>64	0.5	0.25/16	0.75	2	1/32	1	2	0.5/32	0.75
	B81g	>64	0.25	0.125/64	1.5	1	0.5/64	1.5	2	1/8	0.625
	B81f	>64	1	0.25/8	0.375	2	1/64	1.5	2	0.5/64	1.25
***Candida parapsilosis* (n=1)**	C4f		0.0625			0.25			0.5		
***Candida krusei* (n=1)**	C6d		0.125			1			2		

Synergy (background colored light green) had a FICI ≤ 0.5. The addition (background colored light blue) had a FICI between 0.5 and 4. Antagonism had a FICI > = 4 [21]

CAMS-CCPM-D: CAMS Collection Center of Pathogen Microorganisms-D

and antifungals deserved further research. According to reports, the combination of triazoles (fluconazole and ITZ) and AMB against *Cryptococcus neoformans* had an additive effect in vitro but only positive interactions in systemic murine cryptococcosis [28]. The combined treatment of POS and AMB significantly reduced the fungal burden of *Cryptococcus neoformans* in infected brains [29]. DOXY is widely used in skin and soft infection [30] and acne vulgaris [31], with few serious adverse reactions. DOXY belongs to the tetracycline class of antibiotics and inhibit the bacterial protein synthesis by binding to the 30S ribosomal subunit [23]. In vitro, synergism for the association of DOXY and antifungals has been reported against *Candida albicans* biofilms, yeasts and moulds [32–34]. We observed no activity of DOXY alone against Mucorales pathogens but the combination of DOXY plus ITZ, DOX plus POS, and DOX plus AMB showed additive interactions. In the hypothesis, the synergy and additive mechanism between AMB and tetracyclines can be explained by (i) the ability of AMB to form pores in the fungal plasma membrane, allowing the tetracycline antibiotics that can inhibit protein synthesis to enter [19]; or (ii) altering sterol metabolism by inhibiting fungal mitochondrial function with tetracycline, resulting in a decrease in ergosterol levels [35]; or (iii) using DOXY as a chelating agent to alter iron homeostasis and reduce ergosterol content in the cell membrane, providing greater fluidity and allowing antifungals to passively diffuse through the plasma membrane [23].

In our current study, DOXY combined with antifungal therapy has advantages in vitro compared to using antifungals alone. Although this difference may not be significant enough, it offers theoretical feasibility advantages for the development and application of medicine. Prior to this,

further research is needed to investigate the potential benefits and mechanisms of combining antibiotics and antifungal drugs in the treatment of mucormycosis, and to validate such clinical application.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Meijie Zhang and Guanzhao Liang. The first draft of the manuscript was written by Meijie Zhang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests On behalf of all authors, the corresponding author states that there is no conflict of interest.

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