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In vitro antifungal activity of MMV Pathogen Box® compounds alone or in combination with antifungal drugs against mucormycosis agents

Fernando Almeida-Silva [a,1,*], Pedro Henrique Tenório-Alvarenga [a,1], Raiane Valle da Costa [a], Rowena Alves Coelho [a], Glauber Ribeiro de Sousa Araújo [b], Rosely Maria Zancopé-Oliveira [a], Susana Frases [b,c], Rodrigo Almeida-Paes [a,c]

[a] Laboratório de Micologia, Instituto Nacional de Infectologia Evandro Chagas (INI), Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brasil
[b] Laboratório de Biofísica de Fungos, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil
[c] Rede Micologia – FAPERJ, Rio de Janeiro, Brasil

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ABSTRACT

Mucormycosis is a severe fungal infection that demands immediate and decisive intervention upon suspicion. The causative agents of mucormycosis exhibit inherent resistance to echinocandins and voriconazole, and their in vitro susceptibility to terbinafine is highly variable and species-specific. Considering these factors and the limitations of currently available antifungal therapies, the identification of novel antifungals with potent activity against mucormycosis is of paramount importance. This study aims to identify compounds from the MMV Pathogen Box® presenting antifungal activity against selected mucormycosis agents and to evaluate their potential synergistic effects when combined with antifungal drugs. A screening of the Pathogen Box® compounds was conducted, isolated or in combination with sub-inhibitory concentrations of amphotericin B, isavuconazole or posaconazole, against a *Rhizopus oryzae* strain. Hits from the screenings were further evaluated against eight Mucoralean strains for minimal inhibitory and fungicidal concentration determinations and to confirm synergistic interactions using the checkerboard method. Ultrastructural studies were performed using scanning electron microscopy. MMV675968 exhibited fungicidal activity against a *R. oryzae* strain. All but one *Rhizopus* spp. strains presented MIC $\leq$ 1 μg/mL, with a geometric mean of 0.78 μg/mL observed across all isolates for this compound, which did not change significantly the cellular structure of this fungus. The combination screening with antifungal drugs revealed six additional compounds potentially active against the *R. oryzae* strain, two of them demonstrated proven synergism through the checkerboard assay. This first study with the MMV Pathogen Box® and Zigomycetes highlights promising new treatment options for mucormycosis in the future.

1. Introduction

Mucormycosis is a serious fungal infection attributed to a group of fungi distributed within the Mucorales order (Panda et al., 2024). These fungi are found throughout the environment, especially in decaying organic matter and soil. They are characterized by their ribbon-like hyphae, which may lack or have few septa (Prakash and Chakrabarti, 2019). The most common genus implicated in Mucormycosis is *Rhyzopus*, but at least 24 other species from 11 genera can also cause this opportunistic mycosis. In particular, the genus *Cunninghamella* exhibits the highest mortality rate among the Mucoralean fungi (Jeong et al., 2019; Prakash and Chakrabarti, 2019).

Rhino-orbital-cerebral mucormycosis is the most common clinical presentation of mucormycosis, followed by cutaneous and pulmonary involvement (Jeong et al., 2019). Disseminated infections (Horiguchi et al., 2022) and gastrointestinal mucormycosis (Addasi et al., 2023) have also been reported. The significance of mucormycosis has heightened during the Coronavirus Disease 2019 (COVID-19) pandemic due to its opportunistic nature, taking advantage of the increased vulnerability of individuals afflicted with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), thereby rendering them more susceptible to secondary infections such as mucormycosis (Huang et al., 2023).

* Corresponding author at: Laboratório de Micologia, Instituto Nacional de Infectologia Evandro Chagas – INI / Fiocruz - Avenida Brasil, 4365 – Manguinhos 21040-360 - Rio de Janeiro – Brazil.
E-mail address: fernando.almeida@ini.fiocruz.br (F. Almeida-Silva).
[1] These authors contributed equally to this study and their names were placed in alphabetical order.

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Therefore, it is crucial for researchers and healthcare practitioners to recognize the potential threat of mucormycosis, particularly in individuals afflicted with COVID-19, where early intervention is imperative (Hussain et al., 2023; Mahalaxmi et al., 2021).

Mucormycosis is strongly associated with immunosuppressed people (Dar et al., 2024; Darazam et al., 2023). Studies have shown a global upsurge in the incidence of mucormycosis, especially after the COVID-19 pandemic (Panda et al., 2024; Pourazizi et al., 2024; Sharma and Nonzom, 2023). Diabetes mellitus is the main risk factor in Asia (Sigera and Denning, 2023), while transplantation is a major driver in the United States (Wu et al., 2023) and Europe (Puerta-Alcalde and Garcia-Vidal, 2021). The mortality rate for mucormycosis is distressingly high, but treatment with antifungal medications and surgery can be effective (Sigera and Denning, 2023). Amphotericin B in high doses is the first-choice treatment of mucormycosis, with posaconazole and isavuconazole being useful alternatives in combatting the disease (Cornely et al., 2019).

The similarities between fungal and animal cells difficult the development of small molecules that specifically target fungal pathogens (Almeida-Paes and Frases, 2023). Additionally, drug discovery is expensive and time-consuming and only about 20 % of drugs that enter clinical trials are ultimately approved for use (Kim et al., 2023). Hence, the concept of drug repurposing emerges as a strategic approach that allows for the recycling of past efforts into new initiatives (Vanzolini and Magnani, 2024). Currently, many antifungal drugs are in development, but commercially available drugs belong to only a few classes, limiting the number of possible combinations for treating infections that respond poorly to conventional treatment (Stover et al., 2023). Therefore, the search for new molecules that act against certain pathogens may contribute to the development of new monopharmacological or combination therapies (Almeida-Paes and Frases, 2023).

To expedite the discovery of new drugs to treat neglected diseases, Medicines for Malaria Venture (MMV) has assembled several small compound collections for antimicrobial testing, including the MMV Pathogen Box®, which contains 400 compounds that have already been tested against a wide range of diseases. This drug collection has been tested against some human pathogenic fungi, such as *Candida albicans* (Vila and Lopez-Ribot, 2017), *Sporothrix* spp. (Borba-Santos et al., 2020), chromoblastomycosis (Coelho et al., 2020) and mycetoma agents (Lim et al., 2022).

Due to the limited availability of drugs for the treatment of mucormycosis, this study aimed to identify MMV Pathogen Box® compounds with potential in vitro antifungal activity against some mucormycosis agents and to evaluate their potential synergistic effects with existing antifungal drugs.

2. Material and methods

2.1. Strains and culture conditions

In this study it was evaluated nine Mucoralean strains deposited at the Pathogenic Fungal Collection of Oswaldo Cruz Foundation (WDCM registration number: 951). Eight strains have clinical origin, being isolated from human mucormycosis patients during the COVID-19 pandemic, and one was isolated from the environment. These species include *Rhizopus oryzae* ($n = 5$), *Rhizopus delemar* ($n = 2$), *Lichtheimia corymbifera* ($n = 1$), and *Cunninghamella elegans* ($n = 1$). All strains were identified after microscopic examination and MALDI-TOF-MS (MALDI Biotyper – Bruker) analysis using the server version 4.1.100 for analyses. The strains were maintained on potato dextrose agar tubes at 37 °C, with weekly subculturing to induce growth of reproductive structures (Dolatabadi et al., 2015).

2.2. Screening for antifungal activity

The 400 small molecules from the MMV Pathogen Box® were screened in order to identify drugs that could inhibit the growth of mucormycosis agents, similarly to other studies from our group (Coelho et al., 2020). The tests were performed in 96-well plates at a final drug concentration of 1 μM in RPMI 1640 medium (Invitrogen, MA, USA), buffered at pH 7.0 with 3-(N-Morpholino) propanesulfonic acid and supplemented with 2 % glucose. A sporangiospore suspension of the *R. oryzae* (316 GAL) strain was prepared in sterile saline and diluted it in RPMI 1640 medium to a final concentration of 5×10^4 sporangiospores/ml in each well containing the compounds. Control wells consisted of RPMI 1640 without drugs or sporangiospores (negative control) and RPMI 1640 with the same sporangiospore concentration and no drug (growth control). Amphotericin B, a reference drug in the MMV Pathogen Box®, served as a fungal growth inhibition control. The plates were incubated for 24 h at 37 °C and visually inspected. This screening was performed in triplicate and further evaluated the drugs that consistently impaired fungal growth across the experiment replicates.

2.3. Minimal inhibitory concentration assay

Minimal inhibitory concentration (MIC) assays were performed in 96-well plates according to the CLSI broth microdilution method M38-A2 (CLSI, 2008). Firstly, it was tested against the nine Mucoralean fungal strains described in Section 2.1 the traditional antifungal drugs amphotericin B (AMB), posaconazole (POS), and isavuconazole (ISA), purchased from Sigma-Aldrich (St. Louis, MO, USA), and then the drugs that consistently inhibited fungal growth in the MMV Pathogen Box® screening described in Section 2.2. The final drug concentrations ranged from 16 to 0.031 μg/ml, and the final inoculum concentration was 5×10^4 sporangiospores/ml. The MIC was defined by visual readings after 24 h of incubation at 37 °C as the lowest drug concentration that resulted in 100 % inhibition of fungal growth (Badali et al., 2021).

2.4. Minimal fungicidal concentration assay

To determine the minimal fungicidal concentration (MFC), 5 μL of the culture medium were transferred from each well without visible fungal growth in the microdilution plates used for MIC determination (described in Section 2.3) to a new plate with Sabouraud 2 % Glucose Agar (Becton Dickinson and Company– BD, Sparks, MD, USA). The plates were incubated at 37 °C for five days. The lowest drug concentration that did not show any fungal growth was defined as the MFC. A MFC/MIC ratio of 1 to 2 suggests a fungicidal compound, while a ratio higher than 2 suggests a fungistatic compound (Hafidh et al., 2011; Coelho et al., 2020).

2.5. Screening for synergic antifungal activity

After screening the MMV Pathogen Box® drugs for their antifungal activity against the *R. oryzae* strain, it was performed a new screening using the same 400 drugs in combination with the three main drugs recommended for the treatment of mucormycosis (POS, ISA, and AMB), similarly to another previous study of our group (Coelho et al., 2022). In this experiment, were used the same concentrations of the MMV Pathogen Box® drugs and fungal inoculum as described above, supplemented the RPMI 1640 medium with the conventional antifungal drugs at a ½ MIC concentration. Once again the *R. oryzae* (316 GAL) strain was used for these screenings. Control wells consisted of RPMI 1640 culture medium only (negative control) and RPMI 1640 with the same sporangiospore concentration, ½ MIC concentration of the antifungal drug, and no MMV Pathogen Box® drug (growth control). Were considered small molecules that did not inhibit fungal growth in the initial screening but inhibited growth in combination with the antifungal drugs for further evaluation. It was also evaluated the viability of the *R. oryzae* strain in the wells without fungal growth transferring 5 μL of the containing from these wells to a Sabouraud 2 % Glucose Agar plate. The plates were incubated at 37 °C for five days and then observed presence

or absence of fungal growth. Combinations that impaired fungal growth in this experiment were categorized as fungicidal, while those that did not were classified as fungistatic.

2.6. Synergism evaluation

The potential synergism between active drugs from the MMV Pathogen Box® and the traditional antifungals used to treat mucormycosis (POS, ISA, and AMB) was performed using a checkerboard assay in 96-well plates. The concentrations of the drugs tested was based on the previously determined MIC values, described in Section 2.3. Serially dilutions of the traditional antifungal drugs were performed from row A to G and the MMV Pathogen Box® drugs from column 2 to 11, resulting in a 7-by-11 checkerboard design. The final concentrations of the standard drugs were 16–0.25 μg/ml (POS and ISA), 8–0.125 μg/ml (AMB), and 4–0.007 μg/ml (MMV Pathogen Box® compounds). It was used the same fungal inoculum and incubation conditions as for the MIC determination. The confirmation of the drug interaction was accessed and classified according to the fractional inhibitory concentration index (FICI). The FICI was calculated using the formula: FICI = (A/MIC(a)) + (B/MIC(b)) where: A = MIC of the traditional antifungal drug in combination; MIC(a) = MIC of the traditional antifungal drug alone; B = MIC of the MMV Pandemic Box® drug in combination; MIC(b) = MIC of the MMV Pandemic Box® drug (b) alone. The FICI values were interpreted as follows: synergism, when FICI $\leq$ 0.5; indifference, when 0.5 < FICI < 4.0; and antagonism, when FICI $\geq$ 4.0 (Coelho et al., 2022; Odds, 2003).

2.7. Scanning electron microscopy

The *R. oryzae* strain was inoculated through excision of fragments from a culture containing spores and/or mycelial fragments of the fungus on a block of Sabouraud Dextrose agar medium supplemented with ½ MIC of the active MMV Pandemic Box® drug. Controls were performed as above, but on Sabouraud Dextrose agar medium without drug supplementation. Blocks of approximately 6 mm^2 and 2 mm thickness were put at the center of a microscope slide and covered with an 18 × 18 mm coverslip (Paul Marienfeld GmbH Co. KG, Germany). A U-shaped glass rod was used as a framework in a Petri dish lined with filter paper soaked in ultrapure water, aiming to maintain a humid atmosphere. This process was conducted with meticulous attention to aseptic technique to avoid contamination. After inoculation, the Petri dish was sealed and then incubated at 25 ºC for 24 to 48 h. Subsequently, the cover slips with the respective cultures were carefully removed and affixed to stubs. For chemical fixation of the samples through vapors, the stubs were placed in a tightly sealed container with a 2.5 % glutaraldehyde solution (EM grade) and 4 % paraformaldehyde (EM grade) (both from Electron Microscopy Sciences, Hatfield, PA, USA) in a 0.1 M sodium cacodylate buffer at pH 7.2 ± 0.1 for 2 days at room temperature. After fixation, the samples were placed in a desiccator for 48 h in order to reduce humidity and mitigate the possibility of "electric charge buildup" when analyzed under a scanning electron microscopy. Following this, the samples were coated through sputter coating with a 10 nm layer of platinum (Pt) using the Q150R Plus (Quorum Technologies, Judges House, United Kingdom). The samples were observed using a Carl Zeiss Evo LS microscope.

3. Results

3.1. Antifungal activity of the traditional antifungal drugs

Table 1 depicts the antifungal susceptibility data of the strains used in this study. In brief, the MIC values of the traditional antifungal drugs ranged from 0.5 to 16 μg/mL to POS, 1–8 μg/mL to ISA and 0.125–8 μg/mL to AMB, which presented the lower geometric mean among the three conventional antifungals (2.94, 4.0, and 2.72 μg/mL respectively). These three traditional antifungal drugs were fungistatic to all tested isolates (MFC $\geq$ 16 μg/mL for all drug/strain combinations).

3.2. Antifungal activity of the MMV pathogen box® compounds

After the conventional screening with the 400 compounds present in the Pathogen Box®, only the small molecule MMV675968 (Supplementary Figure 1) demonstrated fungal growth inhibition against the *R. oryzae* 316 GAL strain. All but one *Rhizopus* spp. strains presented MIC $\leq$ 1 μg/ml. For the other species, it was observed MIC $\geq$ 4 μg/ml for this compound. Nonetheless, the MMV675968 MIC ranged from 0.125 to 8 μg/ml, with a geometric mean of 0.78 μg/mL for all isolates, demonstrating fungicidal effects to *R. oryzae* and *C. elegans* (Table 1).

3.3. Antifungal activity of the MMV pathogen box® compounds in combination with antifungal drugs

The combination screening with antifungal drugs revealed more active drugs against the *R. oryzae* strain. The combination of the MMV688371 and MMV024406 at 1 μM with ½ MIC of the two tested azoles was fungicidal against this strain. The combination of MMV102872 with AMB was also fungicidal, but with the azoles, the combination of this compound was fungistatic. Other fungistatic combinations of compounds with antifungal drugs were also found, for the MMV688978, MMV688943, MMV676558, and MMV595321. The compound MMV675968, which showed fungicidal activity in the conventional screening, when combined with the conventional antifungal drugs presented a fungistatic activity against *R. oryzae* (Fig. 1). It was further tried to evaluate the seven MMV Pathogen Box® compounds that were able to kill *R. oryzae* during the screening in combination with traditional antifungal drugs. Due to technical issues, there were not enough amount of MMV102872, MMV688247, and MMV676558 to perform the checkerboard assay. Table 2 summarizes the antifungal

Table 1
– Distribution of minimal inhibitory concentrations (MIC) of three conventional antifungal drugs and one compound with anti-*Rhizopus oryzae* antifungal activity from the MMV Pathogen Box®.

Strain	Species	MIC				MFC	MFC/MIC	**MFC/MIC Interpretation**
		POS	ISA	AMB	MMV 675,968	MMV 675,968	MMV 675,968	
316 GAL	*Rhizopus oryzae*	1 μg/mL	1 μg/mL	4 μg/mL	1 μg/mL	1 μg/mL	1	Fungicidal
47,814	*Rhizopus oryzae*	2 μg/mL	8 μg/mL	2 μg/mL	0.125 μg/mL	2 μg/mL	16	Fungistatic
50,523	*Rhizopus delemar*	8 μg/mL	8 μg/mL	1 μg/mL	0.125 μg/mL	2 μg/mL	16	Fungistatic
312 GAL	*Rhizopus oryzae*	2 μg/mL	8 μg/mL	2 μg/mL	0.5 μg/mL	8 μg/mL	16	Fungistatic
1,990,702	*Lichtheimia corymbifera*	4 μg/mL	4 μg/mL	8 μg/mL	4 μg/mL	16 μg/mL	4	Fungistatic
44 MC/4	*Cunninghamella elegans*	0.5 μg/mL	4 μg/mL	2 μg/mL	8 μg/mL	16 μg/mL	2	Fungicidal
323 GAL	*Rhizopus delemar*	4 μg/mL	4 μg/mL	2 μg/mL	8 μg/mL	32 μg/mL	4	Fungistatic
47,809	*Rhizopus oryzae*	16 μg/mL	4 μg/mL	4 μg/mL	0.125 μg/mL	1 μg/mL	8	Fungistatic
310 GAL	*Rhizopus oryzae*	4 μg/mL	2 μg/mL	4 μg/mL	0.5 μg/mL	2 μg/mL	4	Fungistatic

Legend: POS = Posaconazole, ISA = Isavuconazole, AMB = Amphotericin B, MIC = Minimal inhibitory concentration, MFC = Minimal fungicidal concentration.

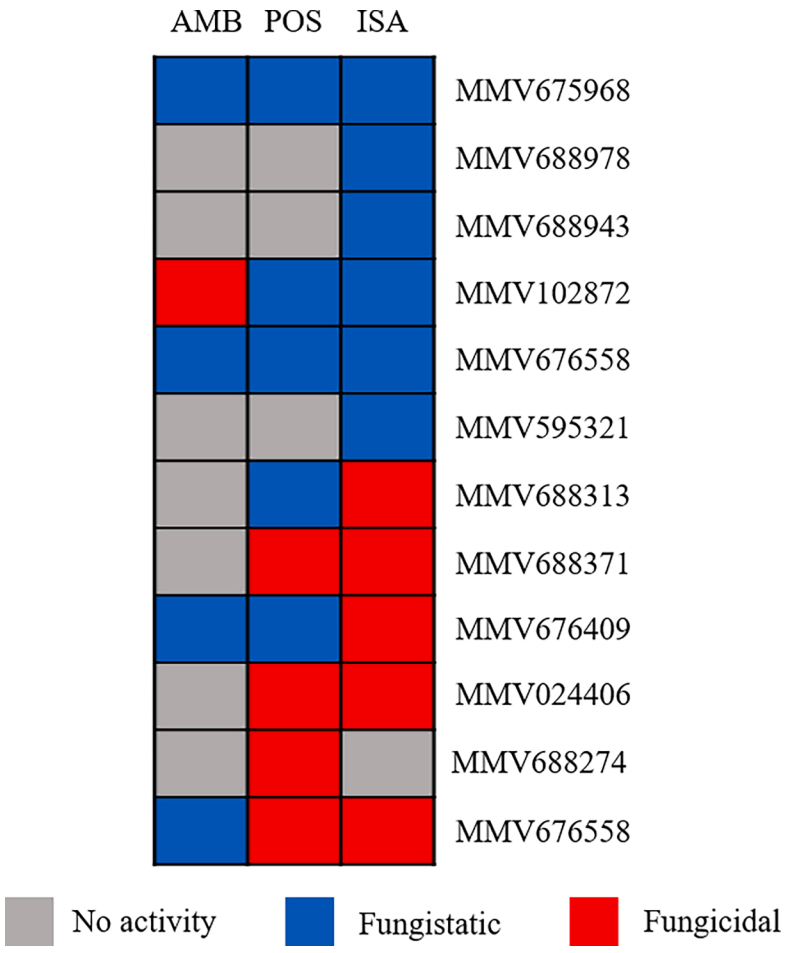

Fig. 1. Drug interactions among the conventional antifungal drugs amphotericin B (AMB), posaconazole (POS), and isavuconazole (ISA) and 11 MMV Pathogen Box® compounds after a screening of this drug collection in combination with half of the minimal inhibitory concentration of the traditional antifungal drugs. Gray squares indicate drug combinations that did not inhibited *Rhizopus oryzae* growth. Blue squares indicate drug combinations that inhibited, but not killed *R. oryzae* (fungistatic combinations). Red squares indicate drug combinations that inhibited and killed *R. oryzae* (fungicidal combinations).

Table 2
Fractional inhibitory concentration index (FICI) of three MMV Pathogen Box® compounds with isavuconazole or posaconazole against the main mucormycosis agent *Rhizopus oryzae*.

Drug combination	MIC of traditional antifungal drug		MIC of MMV Pathogen Box® compound		FICI	Interpretation
	Alone	In combination	Alone	In combination		
ISA/MMV024406	1 μg/mL	0.125 μg/mL	2 μg/mL	0.5 μg/mL	0.375	Synergism
POS/MMV024406	1 μg/mL	0.125 μg/mL	2 μg/mL	0.5 μg/mL	0.375	Synergism
ISA/MMV688371	1 μg/mL	0.25 μg/mL	8 μg/mL	4 μg/mL	0.75	Indifference
POS/MMV688371	1 μg/mL	0.25 μg/mL	8 μg/mL	4 μg/mL	0.75	Indifference
ISA/MMV670409	1 μg/mL	0.25 μg/mL	2 μg/mL	0.25 μg/mL	0.375	Synergism

MIC: minimal inhibitory concentration.
MMV: Medicines for Malaria Venture.
FICI: fractional inhibitory concentration index.
ISA: isavuconazole.
POS: posaconazole.

susceptibility data for the combinations tested using the checkerboard method. The confirmation of the drug interaction showed that compound MMV024406 was synergistic and the compound MMV688371 was indifferent with both azoles (FICI: 0.375 and 0.75, respectively), while the compound MMV670409 was synergistic only with ISA (FICI: 0.375). The MMV688313 compound presented MIC values higher than 4 μg/ml even in combination and therefore it was not further explored.

3.4. Ultrastructural studies

The *R. oryzae* (316 GAL) representative strain submitted to scanning electron microscopy did not show significant differences in the cellular structures analyzed after 24 or 48 h of incubation with the MMV675968 compound (Fig. 2).

4. Discussion

Drug repurposing is a promising approach for increasing the number

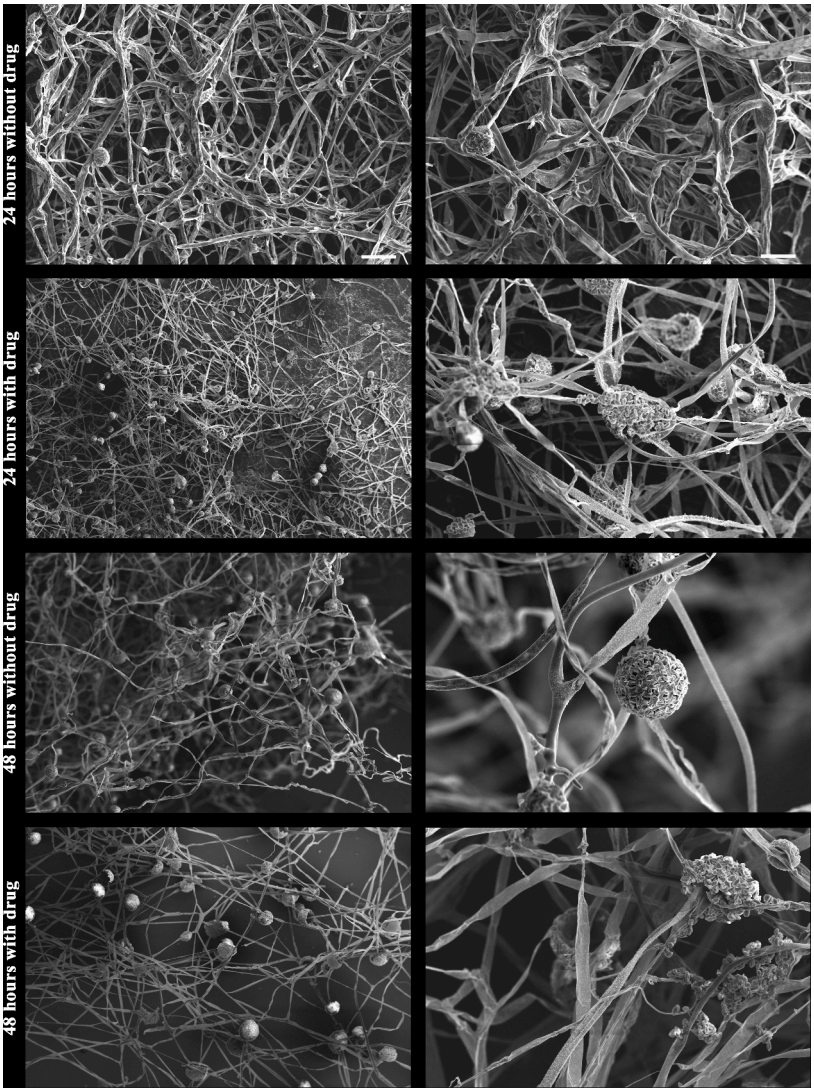

Fig. 2. Scanning electron microscopy of the isolate *R, oryzae* (316 GAL) cultured in the presence and in the absence of the MMV675968 compound at 0.5 μg/mL. Two time points were examined: 24 and 48 h of incubation. In all conditions, coenocytic hyphae and sporangia with sporangiospores are observed, with no surface change among the images. Bars: 20 μm.

of active molecules against a wide range of pathogens and diseases (Jourdan et al., 2020). It is simpler and faster than traditional drug development, and it can be used to develop drug combinations that are more potent than individual drugs (Liu et al., 2021). One strategy for drug repurposing is the screening of drug collections, such as the MMV Pathogen Box®. This drug collection has been tested for some pathogenic fungi such as *Candida auris* (Pan et al., 2023), *Fonsecaea pedrosoi* (Coelho et al., 2020), *Sporothrix brasiliensis* (Borba-Santos et al., 2020), and *Candida albicans* (Vila and Lopez-Ribot, 2017). Another MMV drug collection, MMV Pandemic Response Box®, was tested against three *Rhizopus* species, yielding four compounds with antifungal activity (Xisto et al., 2023). To the best of our knowledge, this is the first time that the MMV Pathogen Box® drug collection is tested against Zygomycetes that cause mucormycosis.

In this study, a single compound from the MMV Pathogen Box®, MMV675968, inhibited the fungal growth of *R. oryzae*, the most important mucormycosis agent, exhibiting fungicidal activity against two Mucoralean strains. It is important to note that the antifungal drugs currently in use for treating mucormycosis were fungistatic against all tested strains. Moreover, MMV675968 presented a geometric mean lower than that observed for the traditional antifungal drugs, suggesting that lower doses of this compounds may be necessary for an effective antifungal activity against the mucormycosis agents. These characteristics are particularly appealing for its potential future use in medications.

Drug combinations have revolutionized the treatment of infectious diseases such as hepatitis C, tuberculosis, AIDS, and cryptococcosis. Notably, the combination of amphotericin B or voriconazole with flucytosine has demonstrated remarkable synergy, particularly in the management of cryptococcosis (Zhao et al., 2024). Therefore, it was decided to investigate whether other MMV Pathogen Box® compounds may act synergistically against the mucormycosis agents, as observed previously with some chromoblastomycosis and phaeophyphomycosis agents (Coelho et al., 2022). Curiously, the combination of MMV675968 with the three antifungal drugs used to treat mucormycosis exhibited fungistatic activity against the same strain that this compound isolated had previously shown fungicidal effects. Exposure to fungistatic antifungals, especially at sub therapeutic concentrations, is a major predisposing factor for the acquisition of resistance (Roilides and Iosifidis, 2019), while fungicidal compounds are less associated with antifungal tolerance (Fisher et al., 2022). Therefore, it is more advantageous to use the compound MMV675968 alone, especially for *R. oryzae* and *C. elegans*, the most common species in mucormycosis cases and the responsible for the highest mortality rates, respectively. Additionally,

the fungistatic effect of MMV675968 against other mucormycosis agents is comparable to that of the commercially available antifungals used to treat mucormycosis, expanding the therapeutic arsenal for this disease. Its low toxicity (CC50 $>$ 25 μM for the HL60 cell) described by the manufacturer of the MMV Pathogen Box® also supports its use in human therapeutics.

In this study, it was demonstrated that drug combinations with repurposed drugs from the MMV Pathogen Box® may expand the therapeutic options for the main agent of mucormycosis, since a fungicidal effect was observed against this fungus during the screening experiment. When combined with major antifungal drugs, seven MMV Pathogen Box® compounds that were ineffective in the conventional screening showed fungicidal activity against *R. oryzae*. The combination of some compounds such as MMV676558, MMV688313, MMV676409, MMV688274, MMV688371 and MMV024406 with azoles were shown to be fungicidal, while the MMV102872 compound was fungicidal in combination with AMB. The checkerboard assay confirmed the synergic activity of MMV024406 with azoles and MMV670409 with ISA. These results suggest that these two compounds may be further studied to improve the mucormycosis treatment.

The active compounds for the main agent of mucormycosis herein reported also exhibit activity against several other pathogens. MMV675968 demonstrated activity against *Trypanosoma brucei* (Dize et al., 2022), *Escherichia coli* (Sharma et al., 2023), *Mycobacterium chimaera* (Cantillon et al., 2022), *Vibrio cholerae* (Kim et al., 2021), *Streptococcus suis* (Songsungthong et al., 2021), *Sporothrix brasiliensis* (Borba-Santos et al., 2020), *Acinetobacter baumannii* (Songsungthong et al., 2019), *Babesia bovis* (Nugraha et al., 2019) and *Toxoplasma gondii* (Spalenka et al., 2018). MMV688371 shows activity against *Trypanosoma brucei* and *Trypanosoma cruzi* (Duffy et al., 2017), while MMV024406 has activity against promastigoste stages of *Leishmania aethiopica* (Tadele et al., 2021). These are promising results, since these compounds may be the basis for new medicines able to combat a milieu of infectious diseases.

Due to the limited number of available antifungal drugs for the treatment of mycoses, most of them targeting ergosterol in the cytoplasmic membrane or its biosynthesis or the fungal cell wall (Sipsas et al., 2018), identifying new active molecules that can act against pathogens in other targets, either alone or in combination, is crucial. Antifungal drugs that act in the cellular membrane or fungal cell wall usually cause ultrastructural alterations in fungi (Yue et al., 2018; Bachmann et al., 2002). The absence of significant modifications in the cell structure of the representative *R. oryzae* strain suggests a mechanism of action different from the traditional antifungals. Unfortunately, it were not able to get good quality images from cultures in broth RPMI-1640, the same culture medium used for MIC determinations, because the fungal structures under these culture conditions were not completely preserved for scanning electron microscopy. This is, indeed, a limitation of this study. Future research is necessary to address this hypothesis and clarify the exact mechanism of action of MMV675968.

Due to the limited MMV Pathogen Box® stocks of some drugs, could not test all drug combinations that presented anti-*R. oryzae* activity shown in Fig. 1, which is another limitation of the current study. In addition, it were not able to evaluate all genus and species that cause mucormycosis. Increased sampling is necessary and studies in other models are essential to further validate the findings of this work. Nevertheless, this study highlights promising new treatment options for mucormycosis in the future.

5. Conclusion

The MMV Pathogen Box® has, at least, three compounds that present antifungal activity against *R. oryzae*. The MMV675968 compound has antifungal activity against this and other mucormycosis agents. The MMV024406 has synergism with two azole drugs, while MMV670409 has synergism with isavuconazole. These three orphan drugs may be useful to enhance the limited existing drug arsenal to treat mucormycosis cases.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2024.100242.

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