Applied Microbiology and Biotechnology (2024) 108:509
https://doi.org/10.1007/s00253-024-13343-8

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Moxidectin elevates *Candida albicans* ergosterol levels to synergize with polyenes against oral candidiasis

Xingchen Ye[1,2] · Yaqi Liu[1,2] · Ding Chen[1] · Binyou Liao[1] · Jiannan Wang[1] · Jiawei Shen[1] · Lichen Gou[1] · Yuan Zhou[1,2] · Xinxuan Zhou[1] · Ga Liao[3] · Xuedong Zhou[1] · Jing Zou[2] · Biao Ren[1]

Received: 30 August 2024 / Revised: 18 October 2024 / Accepted: 22 October 2024

Abstract

Candida albicans, the most common opportunistic pathogenic fungus, is also the main pathogenic organism for oral candidiasis. This condition is particularly prevalent among the elderly, children, and individuals undergoing radiotherapy or suffering from HIV. The lack of new antifungal drugs, and drug resistance coupled with the side effects of current antifungal agents have increased the challenges of clinical antifungal therapies. Polyenes, including amphotericin B and nystatin, are clinical fungicidal drugs, however, their side effects and low solubility have limited their clinical applications. Here, we identified that moxidectin, a novel approved antiparasitic agent, could synergize with both amphotericin B and nystatin to inhibit the growth and biofilm formation of *Candida albicans* including 60 clinical isolates. The transcriptome and RT-PCR analysis indicated that moxidectin activated the biosynthesis pathway of ergosterol, the direct target of polyenes, further being verified by the loss of the synergistic activities with polyenes against ergosterol pathway mutants, including *Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11*. Moxidectin was then confirmed to elevate the ergosterol biosynthesis levels of *C. albicans* and enhance the binding between cells and polyenes. In a mouse oral candidiasis model, moxidectin combined with low dosages of polyenes to significantly reduce the infection area, colonization of *C. albicans* and the inflammatory degree of tongue mucosa. Our study originally demonstrated that moxidectin could activate the ergosterol biosynthesis then elevate the ergosterol contents to enhance the antifungal effects of polyenes against *C. albicans* and its infections. Moxidectin can serve as the candidate potentiator of polyenes for further clinical practice.

Key points

- *Moxidectin synergized with polyenes against Candida albicans.*
- *Moxidectin activated the ergosterol biosynthesis of Candida albicans.*
- *Moxidectin combined with polyenes to effectively combat oral candidiasis in mice.*

Keywords Antifungal · *Candida albicans* · Polyenes · Moxidectin · Ergosterol

Introduction

Candida albicans, a prevalent opportunistic fungal pathogen, has been classified as the critical group among the first fungal priority listed by World Health Organization. (Katsipoulaki et al. 2024). *C. albicans* could lead to superficial infections and potentially fatal systemic infections among low-immunity populations (Calderone et al. 2009). The mortality rate of *C. albicans* associated candidiasis may over 30% in ICU patients (Hellstein and Marek 2019). *C. albicans* can be detected in 30–60% of healthy individuals oral cavity (Vila et al. 2020) and is also the main pathogenic organism of oral candidiasis (Jham et al. 2007). Due to the extensive application of drugs including antibiotics,

✉ Jing Zou
zoujing@scu.edu.cn

✉ Biao Ren
renbiao@scu.edu.cn

[1] State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases & West China School of Stomatology, Sichuan University, Chengdu 610041, China

[2] Department of Pediatric Dentistry, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China

[3] Department of Information Management & Department of Stomatology Informatics, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, Sichuan, China

Published online: 11 November 2024

glucocorticoids, and immunosuppressants, as well as an increase in the number of diabetic patients, AIDS patients, and radiotherapy patients, oral candidiasis is increasing fast and threaten patients' health and quality of life(de Vasconcellos Ferreira et al. 2023; Soysa et al. 2006).

Polyenes are common fungicidal clinical antifungal drugs, mainly including amphotericin B (AmB) and nystatin (Nys), due to their actions on fungal cell membrane (Kristanc et al. 2019). Polyenes can bind to ergosterol, an essential part of the fungal cell membrane, to destroy the cell membrane and lead to the leakage of important substances in the cell or impair the normal metabolism of the cell (Hamill 2013; Sousa et al. 2023). Recently, AmB was also proved to kill fungi by producing extra membranous spongy aggregates to take out ergosterol from the lipid bilayer (Maji et al. 2023; Anderson et al. 2014). AmB is thought to be the "gold standard" in antifungal therapy, with a strong antifungal effect and a broad antifungal spectrum (Lu et al. 2019), and has been used in clinical practice for more than 40 years. Oral amphotericin B has been used as early as 1990 for the prevention of deep-seated fungal infections in patients with hematologic neoplasms and oropharyngeal candidiasis, the most common opportunistic infection in patients with HIV (Kimura et al. 1990; Hood et al. 1998). Amphotericin B suspension also remains a useful therapy in topical therapies in azole-resistant oropharyngeal candidiasis(Grim et al. 2002). In addition to oropharyngeal candidiasis, amphotericin B is often administrated in the treatment of patients with deep

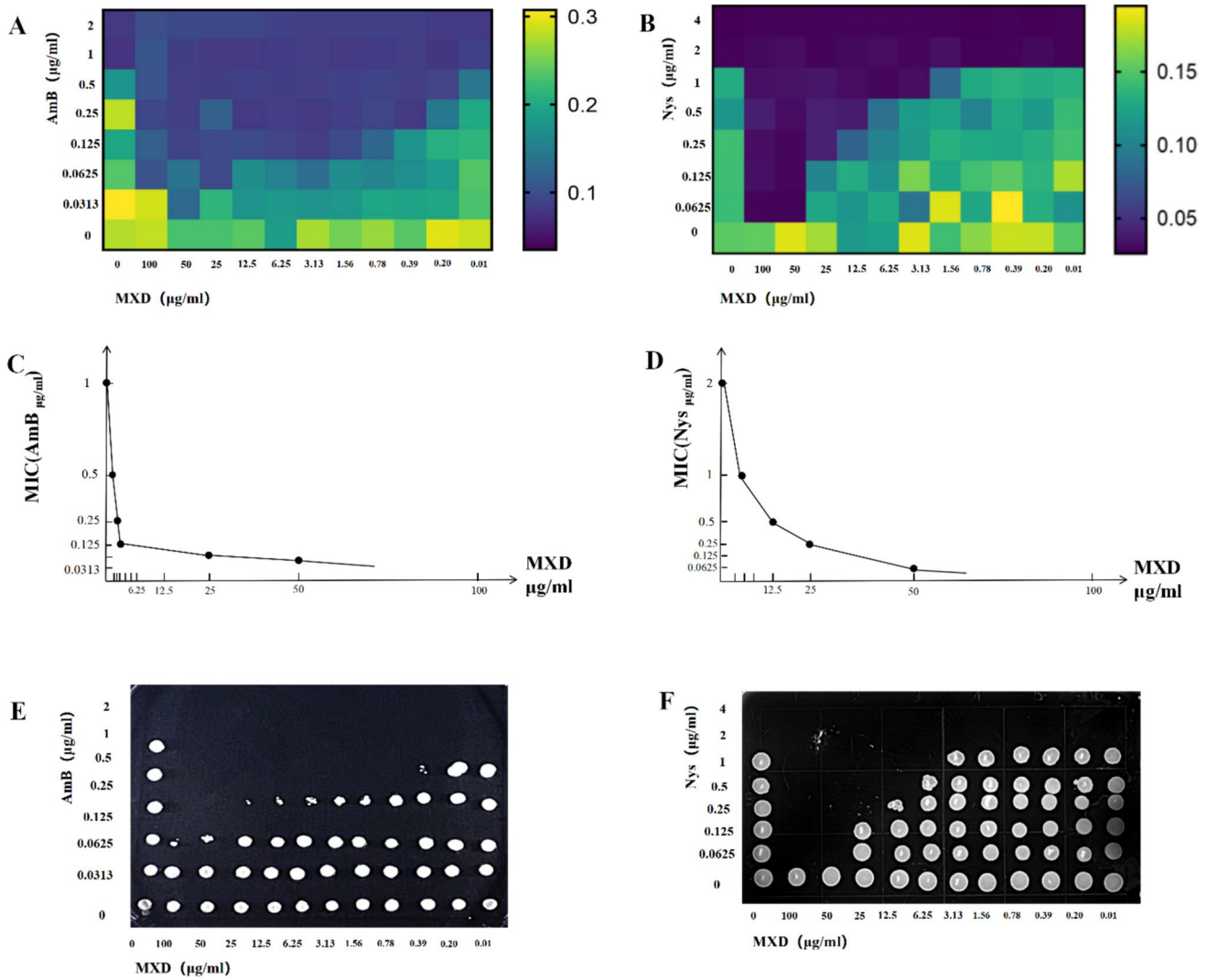


Fig. 1 Moxidectin synergized with amphotericin B and nystatin against the growth of *C. albicans*. *C. albicans* SC5314 were treated with amphotericin B (AmB), nystatin (Nys) and their combinations with moxidectin (MXD) at different concentrations. **(A, B)** MIC turbidimetric thermogram of AmB and Nys in combination with MXD; **(C, D)** combinational MIC curve of AmB and Nys in combination with MXD; **(E, F)** MFC plots of AmB and Nys in combination with MXD

Table 1 Combinational effects between moxidectin (MXD), amphotericin B (AmB) and nystatin (Nys) against clinical isolates *Candida albicans* 1150-1209

Isolates	MICs(μg/ml)					FICI
	AmB	**Nys**	**MXD**	**AmB + MXD**	**Nys + MXD**	
1150	1	4	> 100	0.25 + 25	0.5 + 25	< 0.5
1151	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1152	1	4	> 100	0.25 + 25	1 + 25	< 0.5
1153	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1154	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1155	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1156	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1157	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1158	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1159	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1160	2	4	> 100	0.25 + 25	0.5 + 25	< 0.5
1161	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1162	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1163	1	4	> 100	0.25 + 25	1 + 25	< 0.5
1164	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1165	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1166	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1167	2	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1168	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1169	2	2	> 100	0.5 + 25	0.5 + 25	< 0.5
1170	2	2	> 100	0.5 + 25	0.5 + 25	< 0.5
1171	2	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1172	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1173	1	4	> 100	0.25 + 25	1 + 25	< 0.5
1174	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1175	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1176	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1177	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1178	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1179	2	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1180	2	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1181	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1182	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1183	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1184	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1185	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1186	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1187	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1188	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1189	2	4	> 100	0.25 + 25	0.5 + 25	< 0.5
1190	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1191	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1192	2	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1193	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1194	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1195	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1196	2	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1197	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1198	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5

Table 1 (continued)

Isolates	MICs(μg/ml)					FICI
	AmB	**Nys**	**MXD**	**AmB + MXD**	**Nys + MXD**	
1199	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1200	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1201	2	4	> 100	0.5 + 25	1 + 25	< 0.5
1202	2	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1203	2	4	> 100	0.25 + 25	0.5 + 25	< 0.5
1204	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1205	2	2	> 100	0.5 + 25	0.5 + 25	< 0.5
1206	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1207	2	2	> 100	0.5 + 25	0.5 + 25	< 0.5
1208	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5
1209	1	2	> 100	0.25 + 25	0.5 + 25	< 0.5

fungal infections(Wei et al. 2003), especially in immunocompromised patients(Cai et al. 2023; Lee et al. 2024). And Nystatin, a topical and oral antifungal agent with fungicidal activity against various yeasts and *Candida albicans*, is widely used to treat cutaneous and oropharyngeal candidiasis. It is available in various forms, such as oral suspensions, topical creams, and oral lozenges(Lyu et al. 2016). Nystatin cannot be absorbed from the gastrointestinal tract when taken orally. Therefore, topical use of nystatin is considered to be the most common route of administration in dentistry because of minimal systemic exposure(Rai et al. 2022). In addition, Nystatin plays an important role in the prophylaxis of oral and systemic candidiasis in infants and immunocompromised patients (e.g., people suffering from AIDS, cancer and organ transplant recipients) (Lyu et al. 2016). However, both AmB and Nys have shown their side effects during their clinical applications, such as AmB has serious liver and kidney toxicity (Karimzadeh et al. 2013), and Nys can lead to nausea, vomiting, gastrointestinal distress and other symptoms (Semis et al. 2013).

The synergistic combination of different drugs has been demonstrated to be a practical strategy to expedite drug development (Zhu et al. 2021), as the synergistic drug combinations can improve drug efficacy, reduce the development of drug resistance, application of drug dosages and drug toxicity and side effects (Nivoix et al. 2006). The synergistic potentiators have shown potent activities to enhance the efficacy of polyenes and reduce their application dosages against *C. albicans* infections(Zhu et al. 2021).

Moxidectin (MXD), a broad-spectrum anthelmintic, is widely used in a variety of animals to prevent and control gastrointestinal nematodes and body parasites, with high efficiency, long-lasting effect, and safety properties (Ménez et al. 2012). It was approved by FDA in 2018 to treat human onchocerciasis, commonly referred to "river blindness", caused by *Onchocerca volvulus* (de Moraes and Geary 2020). The antiparasitic mechanism of MXD is to bind to the glutamate-gated chloride ion channel, increasing the inward flow of chloride ions, and in turn induce the hyperpolarization of neuronal resting potential to cause the paralysis and death of parasite (Turner et al. 2015). In mammals, gamma-aminobutyric acid (GABA) is served as the primary neurotransmitter in the central nervous system (Xu et al. 2004). The existence of the blood–brain barrier brings difficulty for MXD to enter the central nervous system, therefore MXD is proved to be with high degree of safety (Turner et al. 2015; Hürlimann et al. 2023).

To identify candidate molecules to potentiate antifungal polyenes and reduce their toxicities, we found that MXD could synergized with both AmB and Nys to increase their antifungal efficacy at low dosages. Therefore, in this study, the synergistic effects and mechanisms between MXD and polyenes against *C. albicans* and its infection were investigated.

Materials and methods

Chemicals

Amphotericin B (CAS 1397–89-3, Purity 98.00%), Nystatin (CAS 1400–61-9, Purity 97.57%) and Moxidectin (CAS 113507–06-5, Purity 99.49%) were purchased from ApexBio, America. Filipin complex (CAS 11078–21-0, Purity 98.00%) was purchased from Shanghai Yuanye Bio-Technology, China. All the chemicals were solubilized in dimethyl sulfoxide (DMSO) (Merck, China) and stored at -20 °C before use.

Strains and culture conditions

Details of strains used in this study were listed in Table S1. *Candida albicans* SC5314 (ATCC MYA-2876). Clinical isolates of *C. albicans* and gene knockout strains wide type strain (CAF2-1, WT), *Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11* were obtained from the State Key Laboratory of Oral Diseases, West China Hospital of Stomatology (Wang et al.

2024; Zhou et al. 2018). All strains were preserved in liquid nitrogen and cultivated on YPD solid medium (2% peptone, 1% yeast extract, 2% glucose and 2% agar) at 35 °C overnight.

Antifungal susceptibility testing

The antifungal activities of AmB, Nys and MXD against *C. albicans* SC5314, clinical strains and WT, *Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11* were tested in 96-well plates (Nest, Wuxi, China) as previously mentioned (Zhu et al. 2021). The concentrations of AmB were ranged from 2 to 0.0313 μg/ml, Nys were 4 to 0.0625 μg/ml, MXD were 100 to 0.0975 μg/ml, by a two-fold dilution, respectively. The 96-well plates were incubated at 35 °C for 24 h. SpectraMax iD5 reader (Molecular Devices, LLC., San Jose, America) was employed to detect the 600 nm for optical density (OD) and the minimum inhibitory concentration (MIC) was the lowest concentration at which there was no fungal growth. The tests were performed with three replicates.

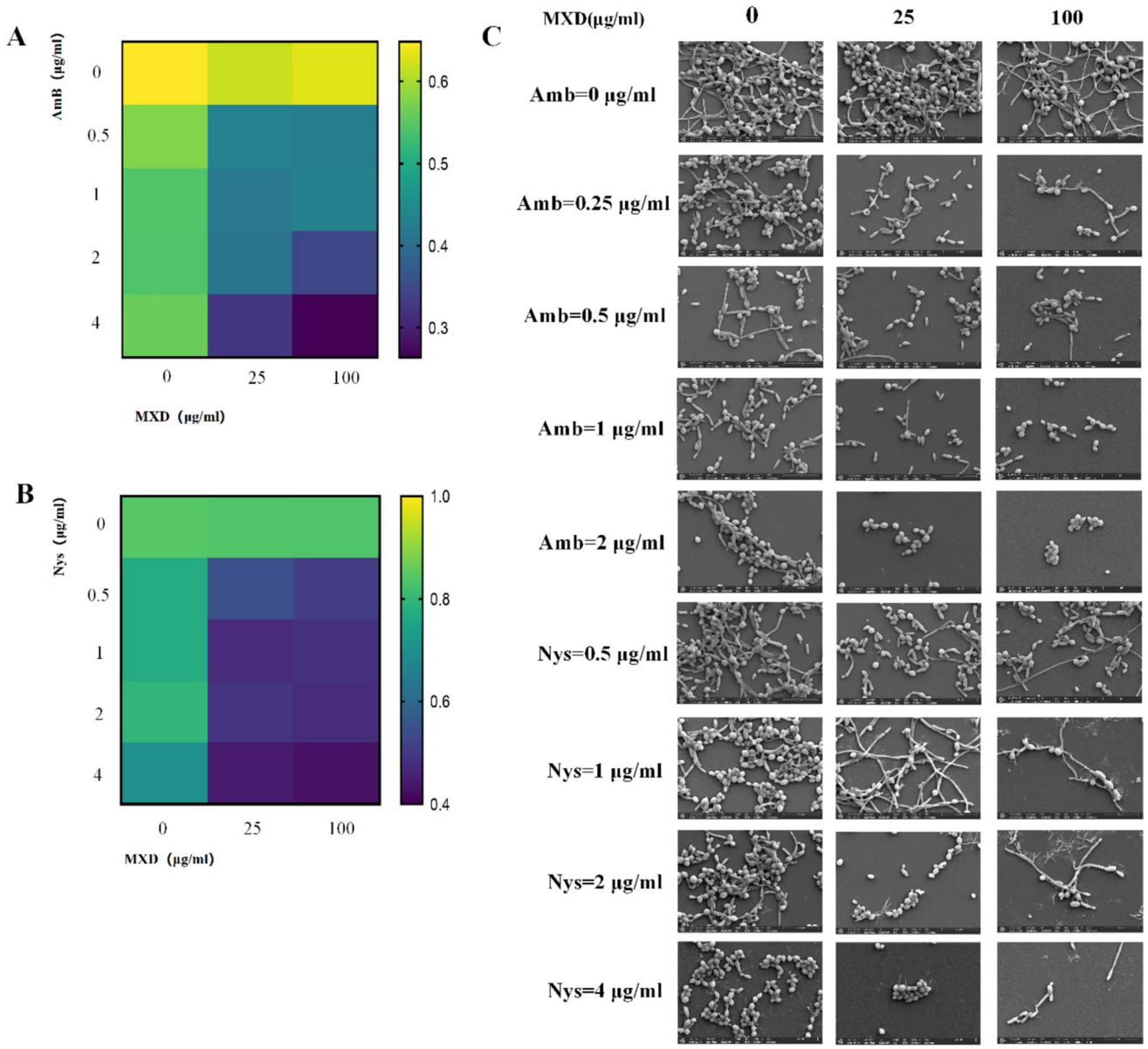
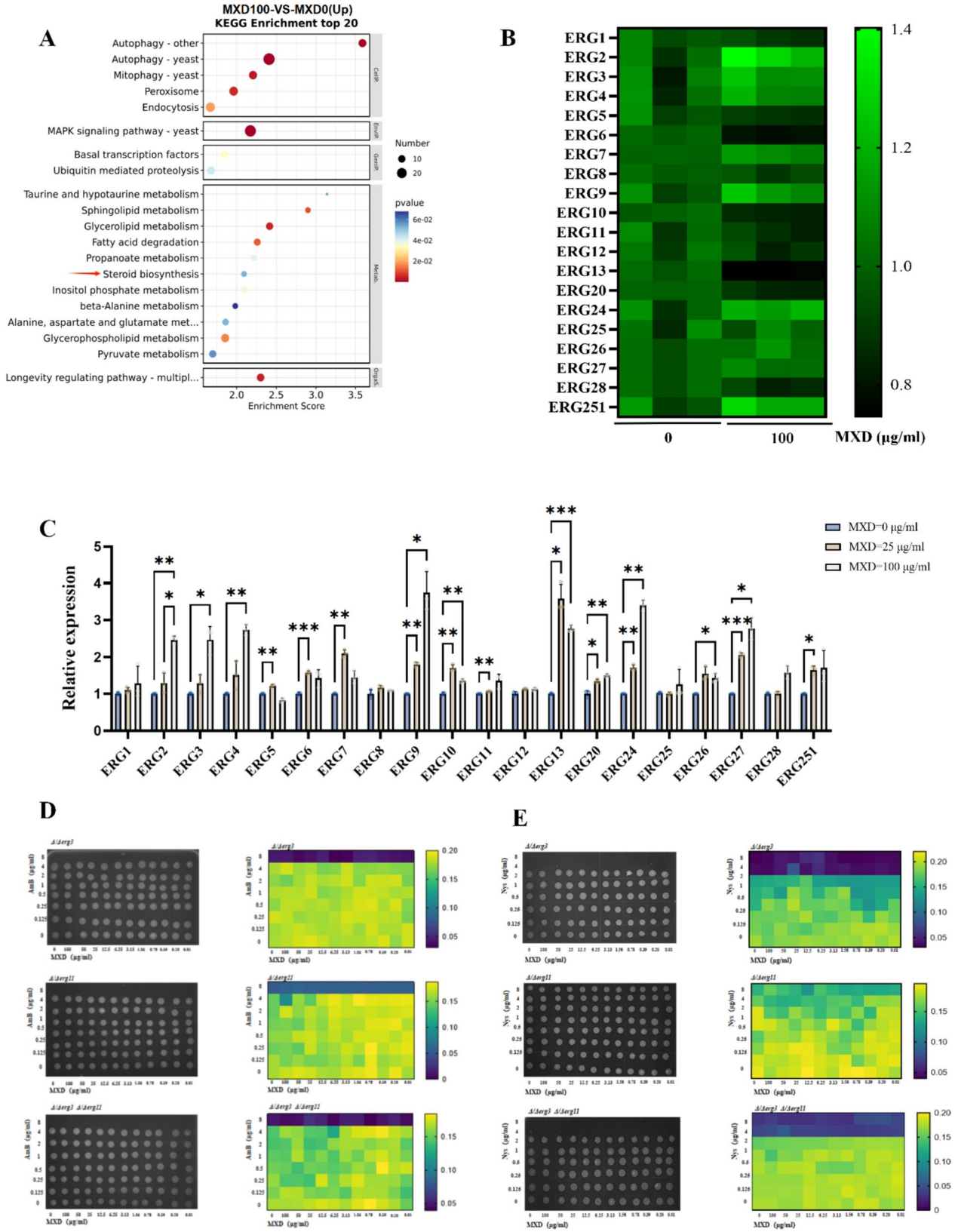


Fig. 2 MXD synergized with polyenes to inhibit the biofilm and filamentous formation of *C. albicans*. The biofilm and filamentous formation of *C. albicans* SC5314 were measured after the treatments with MXD, AmB, Nys and their combinations. **(A)** Crystalline violet turbidimetric thermograms of different concentrations of AmB, MXD and their combinations on *C. albicans* biofilm formation; **(B)** crystalline violet turbidimetric thermograms of different concentrations of Nys, MXD and their combinations on *C. albicans* biofilm formation; **(C)** scanning electron microscopy images of different concentrations of polyenes, MXD and their combinations on *C. albicans* filamentous formation


A
MXD100-VS-MXD0(Up)
KEGG Enrichment top 20
Autophagy - other
Autophagy - yeast
Mitophagy - yeast
Peroxisome
Endocytosis
MAPK signaling pathway - yeast
Basal transcription factors
Ubiquitin mediated proteolysis
Taurine and hypotaurine metabolism
Sphingolipid metabolism
Glycerolipid metabolism
Fatty acid degradation
Propanoate metabolism
Steroid biosynthesis
Inositol phosphate metabolism
beta-Alanine metabolism
Alanine, aspartate and glutamate met...
Glycerophospholipid metabolism
Pyruvate metabolism
Longevity regulating pathway - multipl...
CellP
EnvIP
GenIP
Metab
OrgSys
Number
10
20
pvalue
6e-02
4e-02
2e-02
2.0
2.5
3.0
3.5
Enrichment Score
B
ERG1
ERG2
ERG3
ERG4
ERG5
ERG6
ERG7
ERG8
ERG9
ERG10
ERG11
ERG12
ERG13
ERG20
ERG24
ERG25
ERG26
ERG27
ERG28
ERG251
1.4
1.2
1.0
0.8
0
100
MXD (μg/ml)
C
Relative expression
MXD=0 μg/ml
MXD=25 μg/ml
MXD=100 μg/ml
D
E
MXD (μg/ml)

◂**Fig. 3** MXD activated the ergosterol biosynthesis pathway of *C. albicans* to synergize with polyenes. **(A)** KEGG enrichment of the up-regulation pathways of *C. albicans* SC5314 after 100 μg/ml MXD treatment and the red arrow indicated the "Steroid biosynthesis" pathway; **(B)** heatmap of the expressions of the genes related to the ergosterol biosynthesis in *C. albicans* SC5314 after 100 μg/ml MXD treatment; **(C)** RT-qPCR validation of the relative expressions of ergosterol biosynthesis related genes in *C. albicans* SC5314 treated by 25 and 100 μg/ml MXD; **(D**, **E)** Checkboard antifungal effects of MXD combined with AmB and Nys against *Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11*, left: recovery plates; right: OD600 heatmap of the fungal cell growth overnight

Checkerboard and recovery plate assays

C. albicans SC5314 and WT, *Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11* were cultured as previously described. Samples were spiked in 96-well plates as previously described (Zhu et al. 2021). The fractional inhibitory concentration index (FICI) was used to calculate the drug interactions between polyenes with MXD (Odds 2003). The recovery plate method was employed to furtherly analyze the minimal fungicidal concentration (MFC) of drugs. 1.5 μL of cultures from each well of the 96-well plate were mixed and spotted on YPD solid medium and incubated at 35 °C for 24 h. The tests were performed with three replicates.

Crystalline violet experiment

C. albicans SC5314 was cultured and samples were spiked in 24-well plates as previously described (Liu et al. 2022). The concentrations of AmB were varied from 2 to 0.2 μg/ml, and Nys were 4 to 0.5 μg/ml, MXD were 100, 25, 0 μg/ml, by a two-fold dilution, respectively. After the incubation for 24 h at 35 °C, the supernatant was discarded, then the biofilms were soaked for 3 times in double distilled water. Then the biofilms were dried and fixed for 15 min by adding 1 ml of methanol and soaked once again in distilled water. After drying, each well was stained with 1 ml 0.01% crystal violet for 15 min, dipped and washed again for 3 times, dried and decolorized by adding 1 ml 33% acetic acid to each well. A new plate was taken and 100 μL was aspirated from each well to detect the OD595 value.

Scanning electron microscope assay

The morphology changes of *C. albicans* SC5314 affected by different compounds and combinations were analyzed by scanning electron microscope as previously mentioned (Liu et al. 2022). In brief, *C. albicans* treated with or without compounds were cultured in RPMI1640 for 24 h. The supernatant was discarded, 5% glutaraldehyde was fixed at 4℃ overnight. The fungal cells were rinsed by PBS for 3 times and then subjected to concentration gradient dehydration using different ethanol concentrations ranging from 50 to 100% each for 15 min. Then, the cells were dried, coated with a conductive gold layer and observed by scanning electron microscope (FEI Hillsboro OR, USA).

RNA sequencing and analysis

C. albicans SC5314 was adjusted to 5×10^6 CFU/ml and treated with 100 μg/ml MXD in RPMI 1640 medium for 4 h. Then, collect the fungal cells and preserve them in liquid nitrogen. The subsequent RNA extraction, sequencing and analysis were submitted to Shanghai OE Biotech Co., Ltd (Shanghai, China). The sequencing data were accessible in SRA database (https://www.ncbi.nlm.nih.gov/sra/PRJNA1159473).

RT-PCR assay

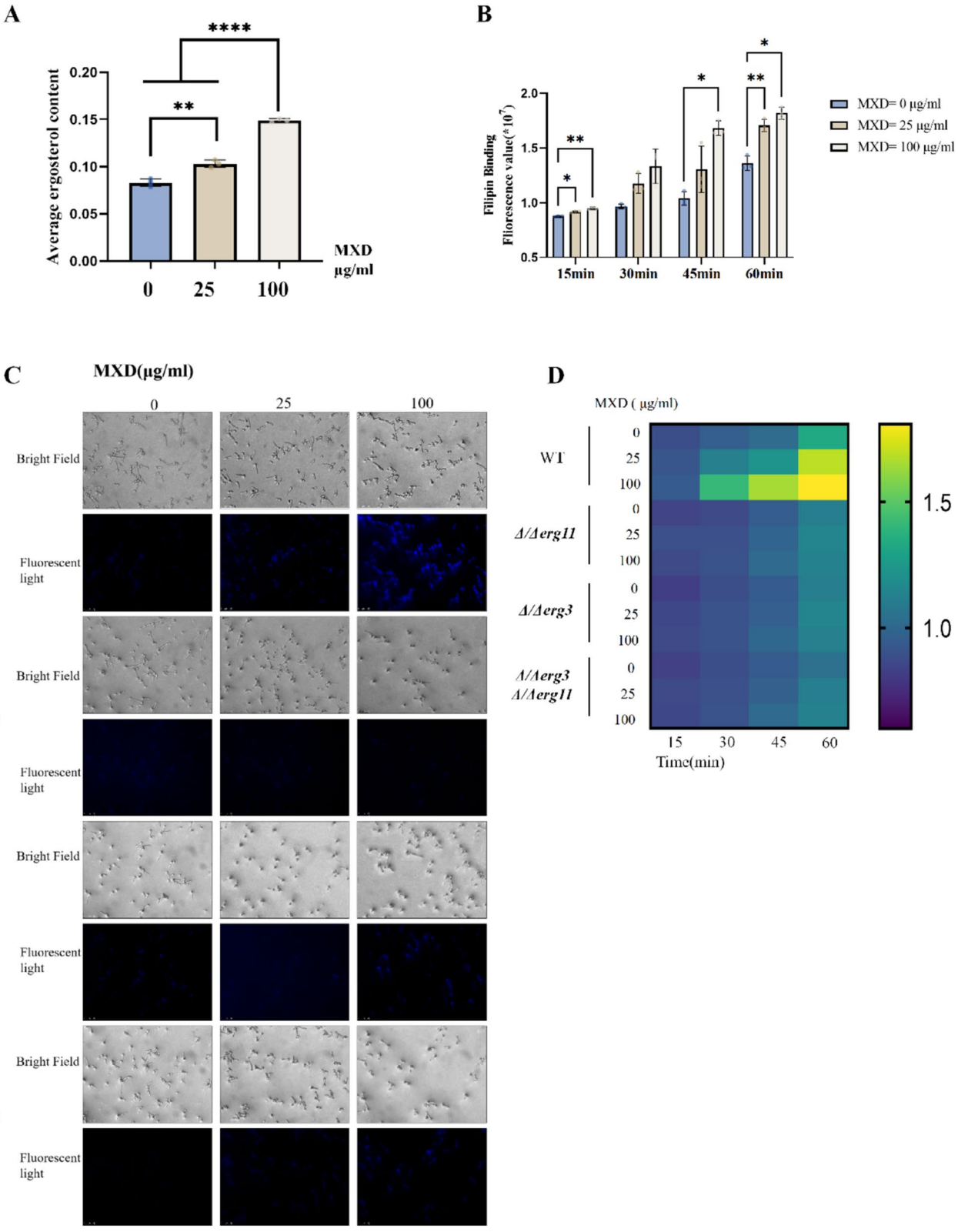
Relative quantification of differentially expressed genes were analyzed by RT-PCR as previously mentioned (Zhu et al. 2021). 2×10^6 CFU/mL *C. albicans* SC5314 cells treated with 25, 100 μg/ml of MXD or DMSO for 4 h were collected. Then the extraction and reverse transcription of RNA followed the instructions of Nucleic Acid Extraction Kit (CWBIO Biotechnology Co., Ltd., China) and PrimeScript™ RT reagent Kit (Takara Bio, China). The primers were listed in Table S2. RT-PCR was performed following the instructions of the TB Green® Premix Ex Taq™ II kit (Takara Bio, China) on a QuantStudio™ 6 Flex (Thermo Fisher, USA). The expression levels of genes relative to calibrators were calculated as $2^{-\Delta\Delta CT}$.

Ergosterol content measurement

The ergosterol content of *C. albicans* SC5314 were measured as mentioned previously (Zhu et al. 2021). Briefly, *C. albicans* was treated with 25, 100 μg/ml of MXD and DMSO as negative control for 4 h. Then the cells were centrifuged at 12 000 rpm 4 °C for 2 min. The weights of the cell pellets were determined after lyophilization. The contents of ergosterol were detected according to the instructions of the micro total cholesterol (TC) content assay kit (Solarbio Life Sciences) and all the experiments were repeated three times.

Filipin binding assay

The fungal cells were cultured and filipin binding assay was performed as previously mentioned (Zhu et al. 2021; Ren et al. 2014). Briefly, 2×10^6 CFU/mL *C. albicans* SC5314 and WT, *Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11* cells were treated with 25, 100 μg/ml of MXD and DMSO for 4 h, then collect *C. albicans* cells and rinse by PBS. After that, treat *C. albicans* cells with 20 μg/ml filipin at 37 °C, 200 rpm for 1 h and collect *C. albicans* cells at different time points, then rinse with PBS. The fluorescence was measured


A
Average ergosterol content
0.20
0.15
0.10
0.05
0.00
****
**
MXD
μg/ml
0
25
100
B
Filipin Binding
Flioresecence value(*10^7)
2.0
1.5
1.0
0.5
**
*
*
**
*
15min
30min
45min
60min
MXD= 0 μg/ml
MXD= 25 μg/ml
MXD= 100 μg/ml
C
MXD(μg/ml)
0
25
100
WT
Bright Field
Fluorescent light
Δ/Δerg3 Δ/Δerg11
Bright Field
Fluorescent light
Δ/Δerg3
Bright Field
Fluorescent light
Δ/Δerg11
Bright Field
Fluorescent light
D
MXD (μg/ml)
WT
Δ/Δerg11
Δ/Δerg3
Δ/Δerg3 Δ/Δerg11
0
25
100
15
30
45
60
Time(min)
1.5
1.0

◂**Fig. 4** MXD raised the ergosterol contents to enhance the binding between *C. albicans* cells and polyenes. **(A)** Ergosterol contents of *C. albicans* SC5314 treated by 25 and 100 μg/ml MXD; **(B)** fluorescence intensity of filipin binding to *C. albicans* SC5314 cells after the treatment with 25 and 100 μg/ml MXD at different time points; **(C)** the bright and fluorescent images of *C. albicans* WT, *Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11* strains treated by 25 and 100 μg/ml MXD combined with filipin at 1 h; **(D)** heatmap of fluorescence intensities of *C. albicans* WT, *Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11* strains treated by 25 and 100 μg/ml MXD combined with filipin at different time points

by SpectraMax iD5 reader with excitation at 340 nm and emission at 480 nm. After 1 h, the *C. albicans* cells were also collected for fluorescence microscopy observation.

The efficacy of MXD combined with polyenes against oral candidiasis in mice

The mouse oral candidiasis model was established using *C.albicans* SC5314 as described previously (Zhou et al. 2018; Moyes et al. 2016; Elahi et al. 2000). In brief, sixty-three 4-week-old female BALB/c mice (Chengdu Dossy Experimental Animal Co,.Ltd., China) were divided into 9 groups ($n=7$ per group): (1) blank control group; (2) 1 μg/ml AmB group; (3) 2 μg/ml Nys group; (4) 100 μg/ml MXD group; (5) 25 μg/ml MXD group; (6) 1 μg/ml AmB + 100 μg/ml MXD group; (7) 1 μg/ml AmB + 25 μg/ml MXD group; (8) 2 μg/ml Nys + 100 μg/ml MXD group; (9) 2 μg/ml Nys + 25 μg/ml MXD group. After euthanasia of the mice, mouse tongues were cut in half longitudinally for quantifying the *Candida* counts on CHROMagarTM Candida agar (CHROMagar, Paris, France) and the subsequent HE and PAS staining (performed by Chengdu Aochuang Biological Company).

Statistical analysis

All statistical analyses were performed using Graphpad prism (version 9.5.0). The infected area of the mouse tongue was analyzed using Image J. Ergosterol content determination, mouse mucosal infection area, and mouse tongue CFU counts were analyzed for differences between their groups using one-way ANOVA. RT-PCR were analyzed with two way ANOVA and Dunnett's multiple comparison test.

Results

Moxidectin synergized with polyenes against *C. albicans*

Amphotericin B (AmB) and nystatin (Nys) showed strong inhibitory activities on the growth of standard *C. albicans* strain SC5314 with MICs at 1 and 2 μg/ml, respectively (Fig. 1A, B), while moxidectin (MXD) had no growth inhibitory capabilities even at 100 μg/ml (Fig. 1A, B), indicating that MXD had no antifungal ability against *C. albicans*. However, MXD significantly sensitized *C. albicans* to AmB and Nys as the MICs could be reduced to 0.0625 μg/ml, respectively, when combined with MXD (Fig. 1A, B, C, D). Meanwhile, the minimal fungicidal concentrations (MFCs) of AmB and Nys were also significantly reduced to 0.125 and 0.0625 μg/ml, respectively (Fig. 1E, F), indicating the strong synergistic antifungal activities between MXD and polyenes. Then 60 clinical isolates of *C. albicans* were employed. The MICs of AmB and Nys against clinical strains were 1–2 μg/ml and 2–4 μg/ml, respectively (Table 1). MXD also did not show inhibitory abilities even at 100 μg/ml, however, it could still synergize with both AmB and Nys to inhibit the growth of all 60 clinical isolates with FICI ≤ 0.5 (Table 1).

The hyphal and biofilm formation were then measured by crystal violet staining and scanning electron microscopy. MXD combined with AmB or Nys significantly inhibited *C. albicans* biofilm formation (Fig. 2A, B) and the hyphal development (Fig. 2C), suggesting the decrease of the *C. albicans* virulence by the combinations between MXD and polyenes.

Moxidectin activated the ergosterol biosynthesis pathway of *C. albicans*

In order to investigate how MXD synergized with polyenes, the transcriptome of *C. albicans* treated with 100 μg/ml MXD was sequenced and analyzed. An alteration in the expression of a multitude of genes was observed.(Fig. S1). The KEGG enrichment analysis of the differentially expressed genes showed that the MXD treatment notably impacted the metabolism pathways, particularly the ergosterol related steroid biosynthesis (Fig. 3A). Since the target of polyenes were ergosterol, the genes associated with ergosterol biosynthesis were analyzed and MXD markedly increased most of the expressions of the essential genes from this pathway (Fig. 3B). Then RT-PCR was performed to ensure the effects of MXD on ergosterol biosynthesis. Both 25 and 100 μg/ml of MXD could upregulated the genes expressions from the ergosterol biosynthesis pathway (Fig. 3C), indicated that MXD could activate the ergosterol biosynthesis to synergize with polyenes, including AmB and Nys. Then *ERG3*, *ERG11*, two key genes from ergosterol biosynthesis pathway, and their double null mutants were involved to furtherly validate that MXD activated ergosterol biosynthesis to synergize with polyene. MXD lost the synergistic activities with both AmB and Nys against *C. albicans Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11* mutants (Fig. 3D, E).

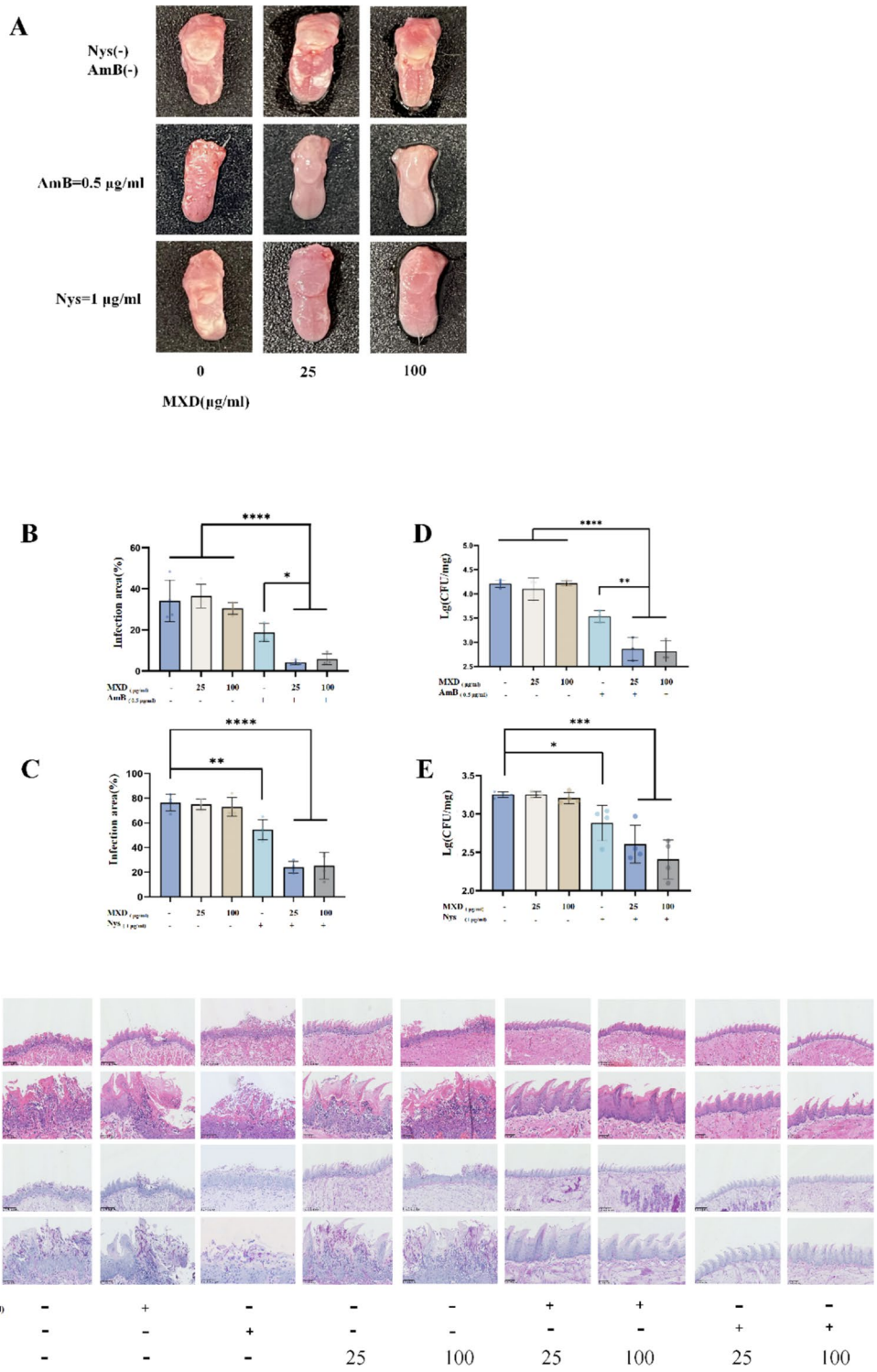


Fig. 5 MXD synergized with polyenes to treat mice oral candidiasis. The mouse oral candidiasis model was established using *C. albicans* SC5314. **(A)** The tongues of mice infected by *C. albicans* from the blank control group, AmB alone group, Nys alone group, MXD alone group, AmB + MXD group, and Nys + MXD group; **(B, C)** the infected area percentages of the tongue mucosa from the mice treated by different drugs and drug combinations; **(D, E)** CFU counts indicated the colonization of *C. albicans* of the tongue mucosa from the mice treated by different drugs and drug combinations; **(F)** HE staining and PAS staining of the tongue mucosa from the mice treated by different drugs and drug combinations

MXD raised the ergosterol contents to enhance the binding between *C. albicans* cells and polyenes

To furtherly confirmed whether the activation of ergosterol biosynthesis by MXD could impact the contents of ergosterol of *C. albicans*, the ergosterol levels were firstly detected. Both 25 and 100 μg/ml of MXD increased the ergosterol levels of *C. albicans* at a dose-dependent manner (Fig. 4A). To validate the increase of ergosterol contents could enhance the binding between polyenes and fungal cell, the fluorescent polyene filipin was employed. Both 25 and 100 μg/ml of MXD could enhance the affinity between *C. albicans* cells and filipin (Fig. 4B, C) in WT, but showed no significant effects on *Δ/Δerg3*, *Δ/Δerg11* and *Δ/Δerg3 Δ/Δerg11* mutants (Fig. 4C, D), indicated that MXD upregulated the ergosterol biosynthesis of *C. albicans* to elevate the ergosterol levels and enhance the binding between fungal cells and polyenes, and in turn to synergize with polyenes.

MXD synergized with polyenes against oral candidiasis in mice

A murine oral candidiasis was then applied to test the synergistic activities between MXD and AmB or Nys against oral candidiasis in vivo. The mice from the blank group and single drug treatment groups, including 0.5 μg/ml AmB, 1 μg/ml Nys, 25 and 100 μg/ml MXD groups, showed the typical *C. albicans* infected white patchy on the tongues (Fig. 5A). However, the combinations between MXD and AmB or Nys significantly reduced the syndromes and infection areas (Fig. 5A, B and C). Then the fungal burden was measured. Both 25 and 100 μg/ml MXD synergized with AmB and Nys to reduce the colonization of *C. albicans* (Fig. 5D, E), in line with the inhibitory activities of MXD and AmB or Nys on the growth and biofilm formation of *C. albicans* in vitro. According to the histological analysis, the tongue mucosa of the mice in the blank control group and single drug treatment groups showed more inflammation, immune cell aggregates, and the surface of the tongue tissue was discontinuity (Fig. 5F), however, the combination groups reduced the inflammation and the epithelia was more integrate and normal (Fig. 5F), indicating that MXD could synergize with polyenes to treat the oral candidiasis. The PAS staining indicated the colonization and invasion of *C. albicans* on the tongue from the blank control group and single drug treatment groups, but in the combination group, no obvious colonization of *C. albicans* was observed (Fig. 5F).

Discussion

In this study, the synergistic antifungal effects against *C. albicans* and oral candidiasis between MXD and polyenes were verified both in vitro and in vivo for the first time. MXD could up-regulate the expressions of genes associated with ergosterol synthesis in *C. albicans* to activate the ergosterol biosynthesis, thereby raising the ergosterol levels of *C. albicans* to elevate the binding between polyenes and cells.

Combination of drugs has gradually become a new popular treatment for various diseases, including antifungal infections, due to the combinational effects in reduction of application dosages, side toxicities of drugs, developments of drug resistance, etc. (Zhu et al. 2023). AmB is a strong fungicidal clinical antifungal agent against various fungal pathogens, however, the side effects and low solubility have challenged its clinical use (Hamill 2013). Therefore, combinations with AmB have been continuously studied in recent years (Zhu et al. 2023). AmB was reported to synergize with azoles, caspofungin, echinocandins and some natural compounds, such as allicin, artemisinin and so on (Larsen et al. 2004; Sugar and Liu 1998; Nivoix et al. 2006; Hossain et al. 2003; Fu et al. 2011; An et al. 2009), while in this study, we found that MXD could also synergistically inhibit *C. albicans* and its infection with polyene drugs by elevating the binding between polyenes and fungal cells through the increase of ergosterol contents, indicating that activation the biosynthesis of ergosterol biosynthesis of fungal cells is a practical target to identify the potentiators for polyene antifungal drugs.

MXD is a macrolide that has been used in animals to fight parasitic infections and it has also been approved to treat onchocerciasis caused by *Onchocercid volvulus* in humans (de Moraes and Geary 2020). There were no reports on the use of MXD in the therapy of antifungal infections and we identified its new mechanisms against *C. albicans* by activating the ergosterol biosynthesis in fungal cells to potentiate the antifungal effects of polyenes. Its synergistic antifungal activities with polyenes on *C. albicans*, including 60 clinical isolates, and oral candidiasis in mice, combined with its clinical safety in humans highly indicate its potential clinical applications in antifungal therapeutics, and more extensive and in-depth clinical studies are needed to continue to validate the activities of their combinations.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00253-024-13343-8.

Author contribution BR, JZ and XDZ contributed to experiment design. XCY, YQL, DC and GL contributed to the conceptualization, data curation, formal analysis, visualization, and writing. XCY, JNW, JWS and BYL contributed to the data curation. XCY, YQL and LCG contributed to experiment development. All authors approved the final manuscript.

Funding This study was supported by National Natural Science Foundation of China (81991500, 81991501, 32071462), Natural Science Foundation of Sichuan province (2024NSFSC0546) and Sichuan Science and Technology Program (2022YFS0285).

Data availability All data generated or analyzed during the study are included in the manuscript.

Declarations

Ethical approval All procedures were conducted in accordance with the "Guiding Principles in the Care and Use of Animals" (China) and were approved by the Ethics Committee of West China Stomatology Hospital of Sichuan University(WCHSIRB-D-2023–624).

Conflicts of interest The authors declare no conflict of interest.

References

An M, Shen H, Cao Y, Zhang J, Cai Y, Wang R, Jiang Y (2009) Allicin enhances the oxidative damage effect of amphotericin B against Candida albicans. Int J Antimicrob Agents 33:258–263

Anderson TM, Clay MC, Cioffi AG, Diaz KA, Hisao GS, Tuttle MD, Nieuwkoop AJ, Comellas G, Maryum N, Wang S, Uno BE, Wildeman EL, Gonen T, Rienstra CM, Burke MD (2014) Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol 10:400–406

Cai W, Ruan Q, Li J, Lin L, Xi L, Sun J, Lu S (2023) Fungal spectrum and susceptibility against nine antifungal agents in 525 deep fungal infected cases. Infect Drug Resist 16:4687–4696

Calderone R, Odds FC, Boekhout T (2009) Candida albicans: fundamental research on an opportunistic human pathogen. FEMS Yeast Res 9:971–972

de Moraes J, Geary TG (2020) FDA-approved antiparasitic drugs in the 21st century: a success for helminthiasis? Trends Parasitol 36:573–575

de Vasconcellos Ferreira PM, Gomes M, Almeida A, Cornélio JS, Arruda TJ, Mafra A, Nunes MHS, Salera RB, Nogueira RF, Sclauser JMB, Drummond-Lage AP, Rezende BA (2023) Evaluation of oral mucositis, candidiasis, and quality of life in patients with head and neck cancer treated with a hypofractionated or conventional radiotherapy protocol: a longitudinal, prospective, observational study. Head Face Med 19:7

Elahi S, Pang G, Clancy R, Ashman RB (2000) Cellular and cytokine correlates of mucosal protection in murine model of oral candidiasis. Infect Immun 68:5771–5777

Fu Z, Lu H, Zhu Z, Yan L, Jiang Y, Cao Y (2011) Combination of baicalein and Amphotericin B accelerates Candida albicans apoptosis. Biol Pharm Bull 34:214–218

Grim SA, Smith KM, Romanelli F, Ofotokun I (2002) Treatment of azole-resistant oropharyngeal candidiasis with topical amphotericin B. Ann Pharmacother 36:1383–1386

Hamill RJ (2013) Amphotericin B formulations: a comparative review of efficacy and toxicity. Drugs 73:919–934

Hellstein JW, Marek CL (2019) Candidiasis: red and white manifestations in the oral cavity. Head Neck Pathol 13:25–32

Hood S, Evans J, Bond J, Wilkins E, Denning D (1998) The treatment of oropharyngeal candidiasis in HIV-infected patients with oral amphotericin B suspension. AIDS Patient Care STDS 12:625–627

Hossain MA, Reyes GH, Long LA, Mukherjee PK, Ghannoum MA (2003) Efficacy of caspofungin combined with amphotericin B against azole-resistant Candida albicans. J Antimicrob Chemother 51:1427–1429

Hürlimann E, Hofmann D, Keiser J (2023) Ivermectin and moxidectin against soil-transmitted helminth infections. Trends Parasitol 39:272–284

Jham BC, França EC, Oliveira RR, Santos VR, Kowalski LP, da Silva Freire AR (2007) Candida oral colonization and infection in Brazilian patients undergoing head and neck radiotherapy: a pilot study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 103:355–358

Karimzadeh I, Khalili H, Farsaei S, Dashti-Khavidaki S, Sagheb MM (2013) Role of diuretics and lipid formulations in the prevention of amphotericin B-induced nephrotoxicity. Eur J Clin Pharmacol 69:1351–1368

Katsipoulaki M, Stappers MHT, Malavia-Jones D, Brunke S, Hube B, Gow NAR (2024) Candida albicans and Candida glabrata: global priority pathogens. Microbiol Mol Biol Rev 88:e0002123

Kimura I, Kuramoto J, Yahata Y, Irino S, Takahashi I, Sezaki T, Tsubota T, Watanabe Y, Hara M, Hino N (1990) A co-operative study on prophylactic effect of oral administration of high-dose amphotericin B syrup for systemic fungal infection in patients with hematological neoplasms. Chugoku-Shikoku Study Group of Mycosis with Hematologic Disease. Gan to Kagaku Ryoho 17:1027–1032

Kristanc L, Božič B, Jokhadar ŠZ, Dolenc MS, Gomišček G (2019) The pore-forming action of polyenes: from model membranes to living organisms. Biochim Biophys Acta Biomembr 1861:418–430

Larsen RA, Bauer M, Thomas AM, Graybill JR (2004) Amphotericin B and fluconazole, a potent combination therapy for cryptococcal meningitis. Antimicrob Agents Chemother 48:985–991

Lee JSF, Cohen RM, Khan RA, Burry J, Casas EC, Chung HY, Costa LH, Ford N, Galvao DLN, Giron N, Jarvis JN, Mondal M, Odionyi JJ, Casas CP, Rangaraj A, Rode J, Ruffell C, Sued O, Ribeiro I (2024) Paving the way for affordable and equitable liposomal amphotericin B access worldwide. Lancet Glob Health 12:e1552–e1559

Liu Y, Wang Z, Zhou Z, Ma Q, Li J, Huang J, Lei L, Zhou X, Cheng L, Zou J, Ren B (2022) Candida albicans CHK1 gene regulates its cross-kingdom interactions with Streptococcus mutans to promote caries. Appl Microbiol Biotechnol 106:7251–7263

Lu Y, Zhou Z, Mo L, Guo Q, Peng X, Hu T, Zhou X, Ren B, Xu X (2019) Fluphenazine antagonizes with fluconazole but synergizes with amphotericin B in the treatment of candidiasis. Appl Microbiol Biotechnol 103:6701–6709

Lyu X, Zhao C, Yan ZM, Hua H (2016) Efficacy of nystatin for the treatment of oral candidiasis: a systematic review and meta-analysis. Drug Des Devel Ther 10:1161–1171

Maji A, Soutar CP, Zhang J, Lewandowska A, Uno BE, Yan S, Shelke Y, Murhade G, Nimerovsky E, Borcik CG, Arango AS, Lange JD, Marin-Toledo JP, Lyu Y, Bailey KL, Roady PJ, Holler JT, Khandelwal A, SantaMaria AM, Sanchez H, Juvvadi PR, Johns G, Hageman MJ, Krise J, Gebremariam T, Youssef EG, Bartizal K, Marr KA, Steinbach WJ, Ibrahim AS, Patterson TF, Wiederhold NP, Andes DR, Pogorelov TV, Schwieters CD, Fan TM, Rienstra CM, Burke MD (2023) Tuning sterol extraction kinetics yields a renal-sparing polyene antifungal. Nature 623:1079–1085

Ménez C, Sutra JF, Prichard R, Lespine A (2012) Relative neurotoxicity of ivermectin and moxidectin in Mdr1ab (-/-) mice and effects

on mammalian GABA(A) channel activity. PLoS Negl Trop Dis 6:e1883

Moyes DL, Wilson D, Richardson JP, Mogavero S, Tang SX, Wernecke J, Höfs S, Gratacap RL, Robbins J, Runglall M, Murciano C, Blagojevic M, Thavaraj S, Förster TM, Hebecker B, Kasper L, Vizcay G, Iancu SI, Kichik N, Häder A, Kurzai O, Luo T, Krüger T, Kniemeyer O, Cota E, Bader O, Wheeler RT, Gutsmann T, Hube B, Naglik JR (2016) Candidalysin is a fungal peptide toxin critical for mucosal infection. Nature 532:64–68

Nivoix Y, Zamfir A, Lutun P, Kara F, Remy V, Lioure B, Rigolot JC, Entz-Werlé N, Letscher-Bru V, Waller J, Levêque D, Koffel JC, Beretz L, Herbrecht R (2006) Combination of caspofungin and an azole or an amphotericin B formulation in invasive fungal infections. J Infect 52:67–74

Odds FC (2003) Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother 52:1

Rai A, Misra SR, Panda S, Sokolowski G, Mishra L, Das R, Lapinska B (2022) Nystatin effectiveness in oral candidiasis treatment: a systematic review & meta-analysis of clinical trials. Life (Basel) 12(11):1677

Ren B, Dai HQ, Pei G, Tong YJ, Zhuo Y, Yang N, Su MY, Huang P, Yang YZ, Zhang LX (2014) ABC transporters coupled with the elevated ergosterol contents contribute to the azole resistance and amphotericin B susceptibility. Appl Microbiol Biotechnol 98:2609–2616

Semis R, Kagan S, Berdicevsky I, Polacheck I, Segal E (2013) Mechanism of activity and toxicity of Nystatin-Intralipid. Med Mycol 51:422–431

Sousa F, Nascimento C, Ferreira D, Reis S, Costa P (2023) Reviving the interest in the versatile drug nystatin: a multitude of strategies to increase its potential as an effective and safe antifungal agent. Adv Drug Deliv Rev 199:114969

Soysa NS, Samaranayake LP, Ellepola AN (2006) Diabetes mellitus as a contributory factor in oral candidosis. Diabet Med 23:455–459

Sugar AM, Liu XP (1998) Interactions of itraconazole with amphotericin B in the treatment of murine invasive candidiasis. J Infect Dis 177:1660–1663

Turner HC, Walker M, Attah SK, Opoku NO, Awadzi K, Kuesel AC, Basáñez MG (2015) The potential impact of moxidectin on onchocerciasis elimination in Africa: an economic evaluation based on the Phase II clinical trial data. Parasit Vectors 8:167

Vila T, Sultan AS, Montelongo-Jauregui D, Jabra-Rizk MA (2020) Oral candidiasis: a disease of opportunity. J Fungi (Basel) 6(1):15

Wang J, Shen J, Chen D, Liao B, Chen X, Zong Y, Wei Y, Shi Y, Liu Y, Gou L, Zhou X, Cheng L, Ren B (2024) Secretory IgA reduced the ergosterol contents of Candida albicans to repress its hyphal growth and virulence. Appl Microbiol Biotechnol 108:244

Wei H, Hai W, Wanqing L (2003) Clinical study on liposomal amphotericin B (Ambisome) in deep fungal infections in China. Mycoses 46:24–28

Xu Q, Cobos I, De La Cruz E, Rubenstein JL, Anderson SA (2004) Origins of cortical interneuron subtypes. J Neurosci 24:2612–2622

Zhou Y, Liao M, Zhu C, Hu Y, Tong T, Peng X, Li M, Feng M, Cheng L, Ren B, Zhou X (2018) ERG3 and ERG11 genes are critical for the pathogenesis of Candida albicans during the oral mucosal infection. Int J Oral Sci 10:9

Zhu C, Liao B, Ye X, Zhou Y, Chen X, Liao M, Cheng L, Zhou X, Ren B (2021) Artemisinin elevates ergosterol levels of Candida albicans to synergise with amphotericin B against oral candidiasis. Int J Antimicrob Agents 58:106394

Zhu P, Li Y, Guo T, Liu S, Tancer RJ, Hu C, Zhao C, Xue C, Liao G (2023) New antifungal strategies: drug combination and co-delivery. Adv Drug Deliv Rev 198:114874