Discovery of a Novel Antifungal Countermeasure Targeting Multidrug-Resistant Fungi

*: yding@cop.ufl.edu

Yousong Ding,[1,*] Dipesh Dhakal,[1] Arindam Sarkar,[2] Mathew E. Smith,[3] Shugeng Cao,[4] and Juannan Zhou[2]

[1]Department of Medicinal Chemistry, Center for Natural Products, Drug Discovery and Development, [2]Department of Biology, [3]Department of Plant Pathology, University of Florida, Gainesville, FL

[4]Department of Pharmaceutical Sciences, Daniel K. Inouye College of Pharmacy, University of Hawaii, Hilo, HI

Introduction

Introduction: Invasive fungal infections cause 2.5 million deaths annually, yet therapeutic options remain limited to five major antifungal classes. Their clinical utility is constrained by toxicity, fungistatic activity, intravenous administration, and the growing emergence of resistance, particularly among CDC-recognized pathogens such as *Candida auris*, and *Candida albicans*. Importantly, Global deployment, traumatic injury, intensive medical care, and multidrug resistance create a distinctive fungal threat to military health. Consequently, antifungal agents with novel chemical scaffolds and mechanisms of action are urgently needed.

Results: Here, we report the discovery of pulvinatal, a bioactive natural product isolated from the understudied bird's nest fungus *Nidularia pulvinata*. Its structure was elucidated by NMR and X-ray analysis. Pulvinatal exhibits potent antifungal activity against all five tested *Candida* species and *Cryptococcus neoformans*, with particularly strong activity against *C. auris* (MIC = 0.156 μg/mL), while displaying no-to-low cytotoxicity toward HEK293, HepG2 and A549 cells (up to 100 μg/mL), yielding a favorable therapeutic window. Notably, pulvinatal also retained potent activity against 10 clinical *Candida* isolates.

Mechanistic studies demonstrate that pulvinatal is fungicidal and induces membrane permeability changes in fungal cells. To investigate its cellular mode of action, we combined ultra-high-resolution barcoded bulk quantitative trait locus (BB-QTL) mapping across ~100,000 yeast segregants[1] with HIP and HOP mutant screening. Integrated genetic and phenotypic analyses implicate microtubule-associated processes in pulvinatal activity.

Conclusions: Together, these findings establish pulvinatal as a novel fungal natural product with potent activity against WHO-priority fungal pathogens and provide genetic evidence linking its antifungal activity to microtubule-associated cellular processes.

Results

1. Bioactivity-guided isolation of pulvinatal.

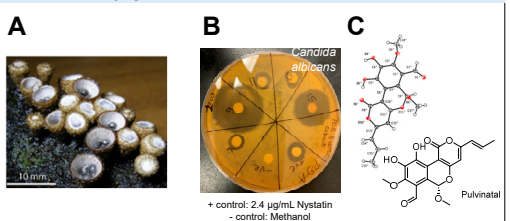

Figure 1. Bioactivity-guided discovery of the antifungal natural product pulvinatal. (A) *Nidularia pulvinata* fruiting bodies. **(B)** Agar diffusion assay against *Candida albicans* used to identify antifungal activity in fungal extracts. **(C)** Structure of pulvinatal determined by NMR spectroscopy and X-ray crystallography.

2. Pulvinatal shows potent antifungal activities toward *Candida* species and clinical isolates.

- Purified pulvinatal was evaluated against multiple yeast pathogens, including *Candida* spp. and *Cryptococcus neoformans*, using the CLSI broth microdilution assay (M27).
- Pulvinatal was further tested against 10 clinical *Candida* isolates obtained from the UF Health Pathology Laboratories Microbiology Laboratory.
- Pulvinatal exhibited potent antifungal activity against both reference strains and clinical isolates.

Table 1. MIC values of pulvinatal against yeast pathogens

Yeast strain	MIC (μg/mL)
C. albicans SC5314	2.5
C. albicans YB3898	2.5
C. tropicalis ATCC 4563	2.5
C. glabrata CBS 138	0.625
C. auris CBS 10913	0.156
C. parapsilosis CBS 604	1.25
S. cerevisiae BY4741	0.313
C. neoformans ATCC 66031	0.625
P. Pastoris X33	< 0.313

Table 2. MIC values of pulvinatal against clinical *Candida* isolates

Clinical Isolates	Pulvinatal	Nystatin	5-Flucytosine	Clotrimazole
1	2.5	0.25	16	1
9	2.5	1	64	0.5
2	5.0	0.5	8	1
3	5.0	0.125	2	0.5
5	5.0	0.25	64	0.25
7	5.0	0.125	32	0.25
8	5.0	2	16	8
4	10	1	4	0.5
6	10	1	4	2
10	10	0.125	32	0.125

3. Phenotypic characterization of the antifungal mechanism of pulvinatal.

A

Cmpd	MIC	MFC	MFC/MIC
pulvinatal	2.5	5	2
Nys	2.5	5	2
Flu	1.25	10	8

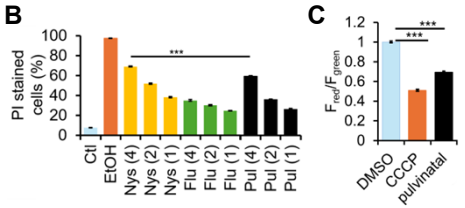

Figure 2. Phenotypic characterization of the antifungal mechanism of pulvinatal against *C. albicans*. (A) Fungicidal vs fungistatic activity of pulvinatal, nystatin and fluconazole against *C. albicans* (n = 3). Nystatin and fluconazole serve as fungicidal and fungistatic control, respectively. **(B)** Membrane integrity effects of compounds (1, 2, 4 x MIC) assessed by PI uptake. Ethanol and untreated cells served as controls (n = 3). **(C)** Mitochondrial depolarization measured with JC-1 after treatment with pulvinatal at 2 x MIC compared to DMSO control (n = 3). Data represent mean ± SD. ***p < 0.001.

4. Barcoded bulk quantitative trait locus (BB-QTL) analysis identified putative chromosomal loci associated with pulvinatal antifungal mechanism

- BB-QTL mapping employs a ~100,000-member yeast segregant library encompassing ~40,000 SNPs, two orders of magnitude greater than previous QTL efforts.
- Barcode sequencing enables measuring the fitness for all genotypes in an ultra-high-throughput manner, capturing both strong and subtle variant effects at unprecedented genomic resolution.
- Large BB-QTL dataset are analyzed by AI/ML approaches (e.g., Borzoi and PolyFun) to identify putative chromosomal loci associated with pulvinatal, which can further pinpoint causal genes and pathways, predict molecular targets, distinguish primary from secondary responses, and reconstruct genetic networks.

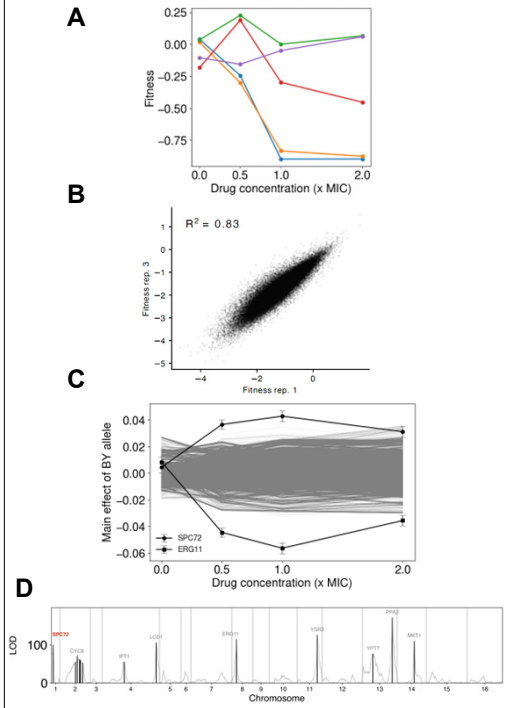

Figure 3. AI/ML powered BB-QTL analysis identified multiple chromosomal loci associated with higher resistance toward pulvinatal. (**A**) Growth response of five representative segregants to serial pulvinatal concentrations (0-2x MIC). (**B**) Fitness estimates show high reproducibility across two biological replicates. (**C**) Genome-wide main effects of SNPs across conditions, with resistance-associated variant in SPC72 along with ERG11. (**D**) SNPs with strong dosage dependence mapped to nearest genes (25) by 2-way ANOVA. SPC72 is shown in red.

5. HIP/HOP mutant screening reveals genetic determinants of pulvinatal sensitivity.

- We acquired both HIP and HOP yeast mutant libraries to complement with BB-QTL analysis.
- A panel of HIP/HOP mutants matching with the majority of 25 genes identified by BB-QTL analysis was prepared to assess their growth fitness (n = 2).
- HIP and HOP mutants of SPC72, ACS1 and GDH3 exhibited significant growth inhibition in the presence of even 1/32 MIC of pulvinatal.
- Among them, the SPC72 HIP and HOP mutants exhibited 2-fold and 8-fold increased sensitivity to pulvinatal, respectively, relative to control strains. SPC72p is associated with spindle pole body and microtubule organization.

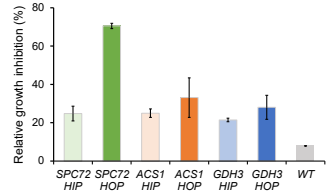

Figure 4. Growth fitness of selected HIP and HOP mutants along with BY4743 wildtype control in the presence of pulvinatal. All strains were treated with 1/8 to 1/64 MIC of pulvinatal for 24 to 48 h. The data represent mean ± s.d. (n = 2) in the presence of 1/32 MIC of pulvinatal.

Table 3. MIC values of pulvinatal against selected HIP and HOP mutants.

Yeast strain	MIC (μg/mL)
BY4743/WT	2
ERG11 HIP	1
SEC20 HIP	1
LCD1 HIP	1
ACS1 HIP	1
ACS1 HOP	0.5
SPC72 HIP	0.5
SPC72 HOP	0.125

Conclusions

1. Pulvinatal represents a novel fungal natural product scaffold discovered from an underexplored fungal source.
2. Pulvinatal exhibits potent antifungal activity against multiple *Candida* species and *Cryptococcus neoformans*, including clinical isolates.
3. Integrated genetic and phenotypic analyses implicate microtubule-associated processes in the antifungal activity of pulvinatal.

Reference

1. Nguyen Ba, A. N., et al. *eLife 2022,* 11:e73983.

Acknowledgement

This work was supported by discretionary funds provided to Y.D. and startup funds provided to J.Z. by the University of Florida.