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Lichen endophyte derived pyridoxatin inactivates *Candida* growth by interfering with ergosterol biosynthesis

Wenqiang Chang, Ming Zhang, Ying Li, Xiaobin Li, Yanhui Gao, Zhiyu Xie, Hongxiang Lou *

Department of Natural Product Chemistry, Key Lab of Chemical Biology of Ministry of Education, Shandong University, No. 44 West Wenhua Road, Jinan City, Shandong Province, China

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ABSTRACT

Background: This study is to characterize the antifungal effects of pyridoxatin (PYR), a small natural product isolated from an endolichenic fungus.
Methods: The susceptibility tests *in vitro* and *in vivo* by using *Caenorhabditis elegans* as an infectious model were performed to evaluate the antifungal efficacy of PYR against *Candida* species. The cytotoxicity of PYR against normal human cells was tested using MTT assay. The transcriptional levels of genes related to sterol synthesis and cell cycle regulation were measured using real-time quantitative PCR (qPCR). The contents ergosterol, squalene, lanosterol were detected by liquid chromatography/tandem mass spectrometry (LC/MS).
Results: PYR was effective against four tested *Candida* species with its minimal inhibitory concentrations (MICs) ranging from 1–4 μg/ml. No obvious cytotoxicity was observed for PYR against normal human cells. PYR inhibited the growth of *Candida albicans*, preventing the biofilm formation. And the antifungal action was independent on efflux pumps. The *in vivo* test showed PYR greatly prolonged the survival of infected *C. elegans*. qPCR results revealed that most of the genes related to sterol biosynthesis were considerably down-regulated in PYR-treated cells. Determination of the sterol content found that PYR inhibited the ergosterol synthesis dose dependently and caused the accumulation of squalene and lanosterol. Moreover, analysis of the structure–activity relationship revealed the heterocyclic hydroxamic acid in PYR was the key group for the antifungal action.
Conclusions: PYR interferes with the ergosterol synthesis to exert antifungal action.
General significance: The elucidated mechanism provides possible applications of PYR in fighting clinical relevant fungal infections.

1. Introduction

Fungal infections, occurring mainly in immunocompromised patients, have caused a considerable problem in clinic settings. *Candida* species ranked predominant nosocomial fungal pathogens, with high rates of morbidity and mortality [1]. Among them, *Candida albicans* and *Candida glabrata* are responsible for approximately 65%–75% of the systemic infection, followed by *Candida parapsilosis* and *Candida tropicalis* [2]. The wild application of broad-spectrum antibiotics, implantable prosthetic devices and immunosuppressive agents together with the emergence of drug resistance accelerates the frequency of fungal infections [1]. The increased incidence of fungal infections highlights the need for new therapeutics with low toxicity.

Ergosterol, an important membrane constituent of fungi, serves as a bioregulator of membrane fluidity, asymmetry and integrity. Proper content of ergosterol is necessary for the growth and normal membrane function of fungal cells [3]. Ergosterol is also involved in several cellular processes including sporulation, pheromone signaling and plasma membrane fusion during mating and endocytosis [4,5]. The antifungal agent fluconazole (FLC) inhibits the activity of sterol 14-demethylase (Erg11p) and the biosynthesis of fungal ergosterol, thereby blocking the proliferation of *C. albicans* [6]. The absence of ergosterol in mammals and suppression of fungal proliferation by a series of ergosterol biosynthesis inhibitors emphasize the importance and utility of ergosterol as an effective target in antifungal chemotherapy.

Natural products have long been regarded as potential sources for the discovery of novel drug leads [7]. Endophytes provide a promising alternative source for biologically active agents discovery owing to the generation of versatile secondary metabolites [8]. Fermentation extracts of *Tolypocladium cylindrosporum*, an endophyte derived from a lichen characterized as *Lethariella zahlbruckner*, displayed promising antifungal activity against *C. albicans* with its effective dose of 8 μg/ml. Further isolation led to the finding of an antifungal active compound pyridoxatin (PYR) from the extract. Although PYR has been previously reported to harbor excellent antifungal activity against *C. albicans* with its MIC of 1.64 μg/ml [9], the potential mechanisms underlying its antifungal action were still poorly understood.

In this study, the antifungal effects of PYR *in vitro* and *in vivo* were investigated and the mode of action was illustrated. Real time quantitative

* Corresponding author at: School of Pharmaceutical Sciences, Shandong University, 44 West Wenhua Road, Jinan 250012, China. Tel.: +86 531 88382012; fax: +86 531 88382019.
E-mail address: louhongxiang@sdu.edu.cn (H. Lou).

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PCR revealed that the ergosterol synthesis pathway was modulated by PYR. Sterol analysis suggested that the reduction of ergosterol content was probably responsible for the antifungal activity mediated by PYR. Furthermore, great reduction of the susceptibility when the N-hydroxyl in the structure was substituted by other groups suggested that the heterocyclic hydroxamic acid moiety was the key functional group for the inhibitory effect.

2. Materials and methods

2.1. Strains and growth conditions

Candida isolates used in this study were shown in Tables 1 and 2. Clinical derived *C. albicans* strains were donated by Professor Qingguo Qi in Shandong University of China. Isolates of *Candida krusei*, *C. glabrata*, and *C. tropicalis* were kindly provided by the Central Hospital of Jinan City. Drug efflux mutant strains [10–12] used in this study were donated from Professor Kim Lewis in Northeastern University of USA. *Candida* strains were propagated in yeast-peptone dextrose (YPD) medium in an orbital shaker at 30 °C and assayed in RPMI1640 medium or plus glucose or serum. *Escherichia coli* and *Bacillus subtilis* were cultured in LB medium. *Caenorhabditis elegans* glp-4; sek-1 strain [13] was purchased from the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. The *C. elegans* was propagated on *E. coli* strain OP50 and cultured using previously described methods [13]. Non-neoplastic, immortalized human prostatic epithelial (RWPE-1) cells were obtained from the American Type Culture Collection (ATCC, Rockwell, MD) and cultured in complete keratinocyte serum-free medium (K-SFM), supplemented with 1% penicillin/streptomycin/amphoterycin B, 50 μg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor (Life Technologies, Barcelona, Spain) as previously described [14]. Normal human bronchial epithelium (HBE) cells were donated from Professor Huiqing Yuan in Shandong University. They were grown in keratinocyte serum-free medium (Life Technologies, Inc. Grand Island, NY) on standard plastic ware (Falcon; Becton-Dickinson, Bedford, MA) at 37 °C in a 5% CO2 atmosphere as previously described [15].

2.2. Antifungal agents

PYR and N-deoxy-pyridoxatin were isolated from *T. cylindrosporum* with its purity over 99% or 93% detected by high-performance liquid chromatography (HPLC) in Agilent 1260 system (Fig. 1). The chemicals were separated on a Eclipse XDB-C18 column (5 μm, 150 mm × 2.1 mm I.D., Agela. Science Inc.,USA) through a 4 mm × 3 mm pre-column (Security Guard C18 cartridge, Phenomenex, Inc.) maintained at room temperature. The mobile phase consisted of methanol/water (70:30, v/v) and was set at a flow rate of 0.80 ml/min. The purity of each compound was calculated based on the peak areas. The structures of PYR and N-deoxy-pyridoxatin have been previously characterized [9,16] and were shown in Fig. 2. FLC was purchased from the Institute of Biopharmaceuticals of Shandong. PYR-D1 was synthesized with following procedures. 1,6-Dibromohexane was synthetically attached with biotin. The derived compound was subsequently conjugated with PYR at hydroxyl group, forming a biotinylated-PYR compound PYR-D1 shown in Fig. 2. The detailed synthetic procedures were described in supplementary materials. The standard samples of ergosterol, squalene and lanosterol used in sterol analysis were purchased from Sigma.

Table 1
C. albicans strains used this study for MICs determination with FLC as positive control.

Organisms	Isolates	MICs (μg/ml) of FLC	MICs (μg/ml) of PYR
C. albicans	SC5314	2	1
	ATCC10231	0.5	1
	YEM30	2	1
	CASA1	1	1
	18b	0.5	2
	25a	0.5	2
	11e	1	1
	11d	1	1
	11f	1	1
	26b	4	2
	23r	8	2
	25f	8	2
	24d	>128	1
	28i	>128	2
C. krusei	CK1	1	2
	CK2	4	1
	CK3	4	1
C. glabrata	CG1	1	1
	CG8	8	2
C. tropicalis	CT2	32	2
E. coli	GB2005		64
B. subtilis	ATCC9372		64

Table 2
C. albicans efflux pumps mutant or hyper-expression strains for MICs determination with FLC as positive control.

Isolates	Characteristics	Reference	MICs (μg/ml) of FLC[a]	MICs (μg/ml) of PYR
CAF2-1	Parent strain for efflux pumps mutants	[30]	2	1
DSY448	*CDR1* mutant	[10]	1	1
DSY653	*CDR2* mutant	[11]	0.5	1
DSY465	*MDR1* mutant	[10]	0.5	1
DSY654	*CDR1* and *CDR2* double mutant	[11]	0.25	1
DSY1050	*CDR1*, *CDR2* and *MDR1* triple mutant	[12]	0.125	1

[a] The MICs of FLC against these mutant strains were tested in this study in parallel with the determination of the MICs of PYR.

2.3. Minimum inhibitory concentration determination

Minimum inhibitory concentration (MIC_{80}) of PYR against *Candida* species was determined by the broth microdilution procedure following the CLSI M27-A3 guidelines [17]. The susceptibility test of efflux pumps mutant strains was also followed as the procedures of MIC determination. The MICs of PYR against bacteria were measured by a broth microdilution assay according to CLSI guidelines [18].

2.4. Cytotoxicity detection by MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT, Sigma) colorimetric assay was used to quantitate RWPE-1 and normal HBE cell proliferation and cytotoxicity in the presence of PYR. Cells (1×10^4 per well) were seeded into 96-well plates as previously described culture conditions [14,15]. After 24 hour incubation, the cells were treated with vehicle, or desired concentrations of PYR for further 24 h. After removing the medium, cells were incubated with 10 μl of MTT for 4 h. The cell growth response to the chemicals was detected by measuring the light absorbance at 570 nm on a plate reader (Bio-Rad Laboratories, Richmond, CA).

2.5. Morphologic transition test of C. albicans

C. albicans strain YEM30 [19] (2×10^5 cells/ml in RPMI1640 medium) was incubated with PYR in 96-well flat-bottomed microtitration plates at 37 °C without shaking. At the indicated time, the cells were photographed with an Olympus IX71 microscope.

2.6. Effect on C. albicans biofilm formation

C. albicans strain YEM30 (1×10^6 cells/ml in RPMI1640 medium) was incubated with PYR in 96-well flat-bottomed microtitration plates