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Identification of LY83583 as a specific inhibitor of *Candida albicans* MPS1 protein kinase

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ABSTRACT

Candida albicans is the most common and virulent fungus causing candidiasis in various parts of the body and can be lethal to immunocompromised patients. All currently known antifungal therapies are drugs which cause serious side effects in the host. An inhibitor specific for fungus survival is an ideal therapeutic. *C. albicans* MPS1 (monopolar spindle 1) has been reported as a kinase essential to its survival. Because CaMps1p shares limited sequence homology with the human ortholog (hMps1p), we screened for a chemical inhibitor in anticipation of finding one with *Candida* specific cytotoxicity. *In vitro* screening using a recombinant catalytic domain of CaMps1p identified LY83583 (6-anilino-5,8-quinolinedione), known as a guanylate cyclase inhibitor, to be blocking CaMps1p kinase activity. In addition to its *in vitro* kinase inhibition, LY83583 reduced the growth rate of *C. albicans*. Finally, we compared the inhibitory activity on CaMps1p and hMps1p among inhibitors against those kinases. LY83583 showed specific inhibition for CaMps1p with no effect on hMps1p activity. Conversely, the CaMps1p activity was not affected by known hMps1p inhibitors. These findings suggest that CaMps1p may well be an ideal target molecule for antifungal therapy.

1. Introduction

Designing antifungal agents is generally more challenging than designing antibacterial agents because of the difficulty in achieving selective toxicity. Moreover, there is a growing problem of drug resistance in fungal infections [1,2]. At present, antifungal therapies are limited to drugs such as amphotericins, azoles and echinocandins that inhibit the growth of *Candida albicans* cells rather than specific virulence processes. These drugs induce disruption of the cell membrane or wall and can be cytotoxic to human cells, leading to serious side effects in the host. In an attempt to find new drug targets, we therefore focused on protein kinases. While drugs targeting protein kinases have been widely used for cancer therapy [3], no such drugs have yet been developed for antifungal therapy.

The *MPS1* gene was first identified in *Saccharomyces cerevisiae* as an essential gene that encodes a protein kinase with functions in spindle pole body duplication and in the mitotic spindle assembly checkpoint maintaining genomic integrity [4,5]. Human Mps1p, also termed TTK, is known as a major mitotic enzyme with roles in chromosome alignment and as a regulator of the spindle assembly checkpoint (SAC), which operates in eukaryotes to prevent the emergence of aneuploidy [6,7]. Due to its central role in mitosis, hMps1p has attracted attention as a target for cancer therapies, and small molecule inhibitors of hMps1p have emerged as anticancer drug candidates [8,9]. Although *C. albicans* Mps1p (CaMps1p) has been shown to be an essential kinase for survival [10], its cellular functions remain unknown. Considering that CaMps1p shares low sequence homology (36% identity) with human homologue, it can be an ideal target molecule to develop anti-*Candida* agents which are expected to have a low harmful effect on the host.

Here we report that LY83583 (6-anilino-5,8-quinolinedione), a guanylate cyclase inhibitor, blocked CaMps1p kinase activity *in vitro* and the growth of *C. albicans*. Furthermore, we found that LY83583 specifically inhibited the kinase activity of fungal CaMps1p without interfering with human ortholog hMps1p activity. These findings suggest that CaMps1p is an ideal target molecule for species-specific antifungal chemotherapeutics.

2. Materials and methods

2.1. Media and basic technique

Table 1 lists the *C. albicans* strains used in this study, and Table 2 lists the primers used in this work. Cells were grown in YPD (1% yeast extract, 2% bacto peptone, 2% glucose adjusted to pH 5.6,

Abbreviations: LY83583, 6-anilino-5,8-quinolinedione; MBP, myelin basic protein; ODQ, 1*H*-[1,2,4]oxidiazolo[4,3-*a*]-quinoxaline-1-one.

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Table 1
Strains used in this study.

Name	Genotype	Reference
TUA4	ura3::imm434 /ura3::imm434 arg4::hisG200/arg4::hisG200	[21]
TUA6	arg4::hisG200/ARG4 RP10::p3HA-ACT1	[22]
MPS1-ARG4	MPS1/mps1::ARG4	This study
MET3-MPS1	mps1::MET3-MPS1/mps1::ARG4	This study

[BD, Franklin Lakes, NJ, USA]), SD–URA, or SD–AU (6.7 g/l yeast nitrogen base without amino acids [BD], 2% glucose, CSM–URA, or CSM–ARG–URA [ForMedium, Norwich, UK]) with shaking. For repression of the *MET3* promoter, methionine and cysteine were added to YPD at the concentration indicated in each figure.

General recombinant DNA procedures were performed as described by Sambrook et al. [11]. *C. albicans* was transformed by the method described by Umeyama et al. [12]. An Applied Biosystems Model 3100 automated capillary sequencer was used for nucleotide sequencing.

2.2. Strain construction

For the construction of the MPS1 heterozygous strain, a 500-bp DNA fragment corresponding to the 5′ or 3′ ends of MPS1 was amplified using primers disMPS1-1 and disMPS1-2 and primers disMPS1-3 and disMPS1-4 to yield DNA fragments disMPS1-A and disMPS1-B, respectively. Two DNA fragments, termed disMPS1-L and disMPS1-R, were amplified using pUC19–ARG4 as a template. DNA fragment disMPS1-A and primers disMPS1-1 and RV-M were used for amplification of disMPS1-L. DNA fragment disMPS1-B and primers disMPS1-4 and M13-47 were used for amplification of disMPS1-R. DNA fragments disMPS1-L and disMPS1-R were simultaneously used to transform the *C. albicans ura–arg*-strain TUA4 to generate MPS1–ARG4.

For the construction of the strain, in which the *MPS1* expression is under the control of *MET3* promoter, a 2.7-kb DNA fragment containing *URA3* marker and *MET3* promoter was amplified using primers, M13-pFA-S1 and MET3p-comp-3′, and as a template to yield URA3–MET3 cassette. Two DNA fragments, referred to as MPS1–URA3-L and MPS1–URA3–MET3p-R, were amplified using URA3–MET3 cassette DNA as a template. DNA fragment disMPS1-A and primers disMPS1-1 and Ura3-3′ new were used for amplification of MPS1–URA3-L. Primers disMET3–MPS1-3′ and Ura3-seqcheck1 were used for amplification of MPS1–URA3–MET3p-R. DNA fragments MPS1–URA3-L and MPS1–URA3–MET3p-R were simultaneously used to transform the *C. albicans MPS1* heterozygous ura-strain MPS1–ARG4 to generate a strain MET3–MPS1.

2.3. Chemical compounds

About 3700 compounds were used for screening, 2400 from a commercial source (AnalytiCon Discovery, Potsdam, Germany), 1000 kindly supplied by Prof. T. Okuda (Tamagawa University) and Prof. Y. Igarashi (Toyama Prefectural University). The SCADS inhibitor kits, about 300 compounds including various kinds of inhibitors were kindly supplied by the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan. LY83583 and staurosporine and 1*H*-[1,2,4] oxidazolol [4,3-*a*] quinoxalin-1-one (ODQ) were purchased from Sigma–Aldrich (St. Louis, MO, USA), SP600125 was from Calbiochem (San Diego, CA, USA), methylene blue was from Wako (Osaka, Japan).

2.4. Purification of CaMPS1p- and hMPS1p-kinase domains

The kinase domains of CaMps1p (CaMps1pΔN, amino acid residues: 516–802) and hMps1p (hMps1pΔN, amino acid residues: 367–637) were amplified by PCR and inserted into the pBAD/Thio-TOPO vector (Invitrogen, Carlsbad, CA, USA), pET21a(+) vector (Novagen, Madison, WI, USA) and, produced in *Escherichia coli* BL21 (DE3). Kinase domains were expressed as fusion proteins containing V5 epitope and His6 tag or His6 tag, respectively, and purified by a nickel affinity column, Ni Sepharose 6 Fast Flow (GE Life-Science, Piscataway, NJ, USA).

2.5. Kinase assay and phosphoprotein detection

For protein kinase assay, 500 ng of purified Mps1p-kinase domains was added to a reaction mixture containing 20 mM HEPES–KOH, pH 7.5, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM DTT and 100 μM ATP, and assayed using 5 mg of exogenous substrate MBP (Millipore, Billerica, MA, USA) for 30 min at 30 °C. Phosphorylated proteins were separated by SDS–PAGE, stained with Pro-Q diamond (Invitrogen) in accord with the manufacture's protocol. Fluorescently stained phosphoprotein was visualized by LAS3000 (FUJIFILM, Tokyo, Japan).

2.6. Growth assay

Overnight cultures of *C. albicans* were diluted 1:150 into YPD medium and incubated at 30 °C. To evaluate the time course of outgrowth, the optical density of 660 nm (OD660) was measured by PhotoMeter (TAITEC) at different time intervals. Inhibitor effect was determined by adding each reagent to the diluted culture at the appropriate concentration and incubated for 9 h. Cell cultures were subjected to 10-fold serial dilution and 1 μl of the dilution was spotted onto a YPD plate. Cells were then cultured for 1 day at 30 °C and photographed by LAS3000.

Table 2
Primers used in this study.

Name	Sequence	Amplified DNA fragment
disMPS1-LFH1	ACTGTGTGGTAGCAAACCGTTTTG	disMPS1-A
disMPS1-LFH2	GTCGTGACTGGGAAAACCCTGGCGTCGTCAAATCCAATCAGTATCTAG	disMPS1-A
disMET3-MPS1-3′	CCTCTAATGCTAAACGTCGTTTGGTTGAATTGTTGCTTGATGGTGACGACCGATGCAGGGAAAAAC TCGTCGCCACATGTGATAATAAATCAT-GTGGCATGTTTTCTGGGGAGGGTATTTAC	MPS1–URA3–MET3p-R
M13-pFA-S1	CGCCAGGGTTTTCCCAGTCACGACGAAGCTTCGTACGCTGCAGGTC	URA3–MET3 cassette
MET3p-comp-3′	CATGTTTTCTGGGGAGGGTATTTAC	URA3–MET3 cassette
Ura3-3′new	CCACCTTTGATTGTAAATAGTAATAATTAC	MET3–URA3-L
Ura3-seqcheck1	TCCTGAGCAACAACCCCATACACAC	MPS1–URA3–MET3p-R

Underlined text represents sequences annealing to vector sequence.