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A new function of isonitrile as an inhibitor of the Pdr5p multidrug ABC transporter in *Saccharomyces cerevisiae*

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Abstract

Pdr5p in *Saccharomyces cerevisiae* is a functional homologue of mammalian P-glycoprotein implicated in multidrug resistance (MDR). In order to obtain useful inhibitors to overcome MDR in clinical tumors, screening of Pdr5p inhibitors has been carried out. We isolated a fungal strain producing Pdr5p inhibitors using our original assay system, and it was classified as *Trichoderma* sp. P24-3. The purified inhibitor was identified as isonitrile, 3-(3′-isocyano-cyclopent-2′-enylidene)-propionic acid, a compound whose carboxyl residue is essential for the inhibitory activity. A non-toxic concentration of the isonitrile (41.5 μg/ml, 255 μM) inhibited Pdr5p-mediated efflux of cycloheximide or cerulenin in Pdr5p-overexpressing cells. In addition, addition of the isonitrile led to accumulation of rhodamine 6G, a substrate of Pdr5p, in the Pdr5p-overexpressing cells. The inhibitory profiles of the isonitrile against S1360 mutants (S1360A and S1360F) of Pdr5p were different from those of FK506 and enniatin. The isonitrile did not influence *PDR5* gene expression and the amount of Pdr5 protein, nor did it inhibit the function of Snq2p, a homologue of Pdr5p. Interestingly, the isonitrile inhibited the function of Cdr1p and Cdr2p, Pdr5p homologues in pathogenic yeast *Candida albicans*. Thus, it was found that the isonitrile shows a different inhibitory spectrum from that of FK506 and enniatin as a potent inhibitor for Pdr5p, Cdr1p, and Cdr2p.

Keywords: *Saccharomyces cerevisiae*; Isonitrile; Pdr5p; Snq2p; Cdr1p; Cdr2p; ABC transporter; Multidrug resistance; FK506; Enniatin

Enhanced efflux of drugs through ATP-binding cassette (ABC) transporters, which is observed in all cells from microorganisms to mammalians, constitutes a major cause of multidrug resistance (MDR). In yeast *Saccharomyces cerevisiae*, the existence of 29 proteins belonging to the ABC transporters has been predicted [1]. Expression of several yeast ABC transporters mediates the pleiotropic drug resistance (PDR) phenotype [2]. The PDR phenomenon in *S. cerevisiae*, mediated by the Pdr5p, is functionally analogous to MDR phenomenon in mammalian cells mediated by P-glycoprotein (P-gp). The Pdr5p interacts with a number of P-gp substrates [3,4] and modulators such as FK506 [5,6], flavonoids [7], and protein kinase C effectors [8]. Therefore, Pdr5p is considered to be an important model protein to study the function of MDR and to search for its inhibitors [9,10].

We succeeded in constructing a highly sensitive and specific assay system for Pdr5p inhibitors in *S. cerevisiae* [11] and screened for microorganisms producing Pdr5p inhibitors. Recently, we reported that enniatin B, B1, and D from *Fusarium* sp. Y-53 exhibit a new function as Pdr5p-specific inhibitors [12]. We also showed that some inhibitory profiles of enniatin were similar to those of FK506, a previously reported Pdr5p inhibitor, and

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the inhibitory activity was competitive with that of FK506.

In this paper, we describe isolation and some characteristics of an isonitrile, a new candidate Pdr5p inhibitor, from *Trichoderma* sp. P24-3.

Materials and methods

Yeast strains, medium, and plasmids. *S. cerevisiae* KHW4 (*Mat a Δpdr5*::*LEU2*, *Δsnq2*::*HIS3*) and KHW3 (*Mat a* Δsyr1/*erg3*::*HIS3*, *Δpdr5*::*LEU2*, *Δsnq2*::*HIS3*) were derivatives of W303-1A [10]. DKY1 (*Mat α leu2 -3*, *-112*, *ura3-52*, *his3-Δ200*, *trp1-Δ901*, *lys2-801*, *suc2-Δ9*, *pdr5-Δ1*::*hisG*, *Mel-*) was a derivative of SEY6210 [13]. Yeast multicopy plasmids, pYI, pYI-*PDR5*, and pYI-*SNQ2*, were gifts from Prof. T. Miyakawa of Hiroshima University [14]. Yeast transformations were performed by the lithium acetate procedure of Ito et al. [15]. Yeast low-copy number plasmid, pKV2, containing *PDR5-lacZ* fusion gene was a gift from W. Scott Moye-Rowley of the University of Iowa [13]. Standard yeast media were used for growth of cells and drug resistance assays [16].

Chemicals. Cycloheximide and rhodamine 6G were purchased from Wako Pure Chemical Industries. Cerulenin and 4-nitroquinoline N-oxide (4-NQO) were purchased from Sigma–Aldrich Chemicals. Ketoconazole was purchased from Funakoshi. FK506 was a gift from Fujisawa Pharmaceutical. Cycloheximide was dissolved in distilled water, cerulenin, rhodamine 6G, and FK506 were dissolved in ethanol, 4-NQO was dissolved in acetone, and ketoconazole was dissolved in DMSO.

Isolation of Trichoderma sp. P24-3 and production of the isonitrile. Strain P24-3 was obtained as a result of a screening involving more than 10,000 microorganisms isolated from soil, using our original assay system for Pdr5p inhibitors in yeast [11]. According to 28S rDNA analysis, the strain was classified as *Trichoderma* sp. P24-3. The medium for Pdr5p inhibitor production consisted of 20% potato extract supplemented with 1% glucose and 0.5% yeast extract (pH 6.8). The strain P24-3 was cultured at 30 °C for 72 h (agitation rate, 350 rpm; aeration rate, 3 L/min) in a 30-L jar fermentor containing 20 L medium.

Methylation. About 20 mg of the purified isonitrile was dissolved in chloroform. The carboxyl residue of the isonitrile was methylated (esterified) with stirring for 3 h at 0 °C by diazomethane, which was prepared from *N*-nitroso-*N*-methylurea and alcoholic KOH. After the reaction, the excess amount of diazomethane was removed in vacuo, yielding colorless liquid. The methylated isonitrile was separated by TLC (solvent; diethyl ether: cyclohexane = 8:2), and the purified compound was extracted with chloroform. Purity of the methylated isonitrile was confirmed by HPLC.

Pdr5p inhibitory assay. Pdr5p inhibitory assay was performed basically as described by us previously [11]. (i) *Agar-well diffusion assay.* Yeast cells grown in SD-uracil medium were spread on a SD-agar plate with or without cycloheximide (0.04 μg/ml final) or cerulenin (0.4 μg/ml final). The sample was applied to a hole (8 mm in diameter) of the plate and was incubated at 30 °C for 48 h. The diameter of the growth inhibitory zone was measured. Inhibitory unit (IU/ml) was calculated using a standard curve [11]. (ii) *Liquid culture assay.* Yeast cells were inoculated into fresh SD-uracil medium (at a cell density of 1×10^5 cells/ml) with or without drugs (cycloheximide, cerulenin, or ketoconazole). Samples were added to the yeast cell suspension and were cultivated at 30 °C for 24 h in a microtiter plate. Absorbance at 660 nm was measured using a microtiter plate reader (MTP-500, CORONA electric, Japan).

Rhodamine 6G accumulation assay. Yeast cells (1.0×10^6 cells/ml) grown for 12 h at 30 °C in SD-uracil liquid medium were treated with or without the isonitrile and then cultivated for 2 h at 30 °C. Rhodamine 6G (final conc. = 1 μg/ml) was added and incubated for 30 min at 30 °C. After washing, the cells were resuspended with 1.0 ml of phosphate-buffered saline (PBS) containing 2% glucose and the fluorescence was visualized using a fluorescent microscope (Nicon Eclipse E-600, Japan).

β-Galactosidase assay. Enzymatic activity of β-galactosidase expressed from *lacZ* gene fused to *PDR5* promoter was measured using *o*-nitrophenyl-β-D-galactoside (*o*-NPG) as a substrate. Yeast cells (DKY2 with low-copy plasmid that includes *lacZ-PDR5* promoter region (~600 bp) containing PDRE [13]) were grown on YEPD medium. The yeast culture ($A_{660} = 1$ (1.0×10^7 cells/ml)) was treated with the isonitrile for 8 h at 30 °C. The collected cells were resuspended with Z-buffer (consisting of 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 7.0) and lysed by vortexing with 45 μl chloroform and 0.1% SDS. The lysed cells were incubated at 30 °C for 10 min with 200 μl of 4 mg/ml *o*-NPG. After centrifugation at 15,000*g* for 20 min at 4 °C, the liberated *o*-NP was measured by absorbance at 420 nm, and β-galactosidase activity (unit) was calculated using the following equation [16]:

$$\text{Unit} = A_{420} \times 1000/A_{660} \times \text{reaction time (min)} \times \text{assay volume} \quad (1)$$

Western blotting analysis. Yeast cells grown in SD-uracil medium were harvested and washed twice with chilled ion-exchanged water. The washed cells were lysed with 150 μl of 1.85 M NaOH–7.5% mercaptoethanol for 10 min on ice. Subsequently, 150 μl of 50% trichloroacetic acid was added to the solution, and the samples were kept for 10 min on ice. After centrifugation at 13,000*g* for 3 min, the precipitates were washed with acetone and dissolved in 50 μl of sample buffer (40 mM Tris–HCl (pH 6.8), 8 M urea, 5% sodium dodecyl sulfate, 0.1 mM EDTA, 1% mercaptoethanol, and 0.01% bromophenol blue). An aliquot of each sample (20 μl) was applied to a 7.5% SDS–polyacrylamide gel after heating at 42 °C for 15 min, and protein bands were transferred to a nitrocellulose membrane. The Pdr5 protein was detected using rabbit anti-Pdr5p antibody and alkaline phosphatase-conjugated anti-rabbit IgG.

Results

Purification of Pdr5p inhibitor from Trichoderma sp. P24-3

For the purification of the Pdr5p inhibitor, culture filtrate (4 L) was concentrated in vacuo and extracted with 1-butanol. The crude extract was applied to a silica gel column (ϕ 30 × 150 mm, Wako Pure Chem.) and eluted with a linear gradient of 10–50% methanol in chloroform. Active fractions were loaded on a CAPCELL PAC UG120 C-18 column (ϕ 4.6 × 250 mm, SHISEIDO Fine Chemicals) using an HPLC system and eluted with 10% acetonitrile in Milli-Q water containing 100 mM triethylamine/acetic acid buffer (pH 7.0). The inhibitor was detected using absorbance at 270 nm and active fractions were extracted with chloroform.

Chemical structure of the methylated isonitrile

The purified inhibitor was stabilized by methylation of its carboxyl residue with diazomethane. The chemical structure of the methyl ester derivative was determined by ^{1}H NMR, ^{13}C NMR, HMQC (INOVA-600 VARIAN), and MS spectrometry (JMS-700 spectrometer).