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Enniatin has a new function as an inhibitor of Pdr5p, one of the ABC transporters in *Saccharomyces cerevisiae*

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Abstract

Pdr5p is one of the major multidrug efflux pumps whose overexpression confers multidrug resistance (MDR) in *Saccharomyces cerevisiae*. By using our original assay system, a fungal strain producing inhibitors for Pdr5p was obtained and classified as *Fusarium* sp. Y-53. The purified inhibitors were identified as ionophore antibiotics, enniatin B, B1, and D, respectively. A non-toxic concentration of each enniatin (5 μg/ml, ~7.8 μM) strongly inhibited a Pdr5p-mediated efflux of cycloheximide or cerulenin in Pdr5p-overexpressing cells. The enniatins accumulated a fluorescent dye rhodamine 123, a substrate of Pdr5p, into yeast cells. The mode of Pdr5p inhibition of enniatin was competitive against FK506, and its inhibitory activity was more potent with less toxicity than that of FK506. The enniatins showed similar inhibitory profile as FK506 against S1360 mutants (S1360A and S1360F) of Pdr5p. The enniatins did not inhibit the function of Snq2p, a homologue of Pdr5p. Thus, it was found that enniatins are potent and specific inhibitors for Pdr5p, with less toxicities than that of FK506.

Keywords: *Saccharomyces cerevisiae*; Pdr5p; ABC transporter; Multidrug resistance; FK506; Enniatin

Multidrug resistance (MDR) protein in cancer cells causes major clinical problems in cancer chemotherapy, because it transports a variety of structurally and functionally unrelated anticancer drugs from the cells [1,2]. Many inhibitors of the MDR protein have been discovered so far, but most of them exhibit cytotoxicity or side effects [3–5]. Therefore, a search for MDR inhibitors with diminished or no cytotoxicity, required for cancer chemotherapy and also useful for elucidation of drug efflux mechanisms by MDR protein, has been under way.

The yeast *Saccharomyces cerevisiae* has about 30 distinct genes encoding ABC (ATP-binding cassette) proteins such as Pdr5p, Snq2p, Yor1p, and Ste6p [6]. Among them, Pdr5p (pleiotropic drug resistance 5 protein) is one of the major sources of drug resistance against various compounds such as cycloheximide, cerulenin, rhodamine 6G, anticancer drugs (doxorubicin, tamoxifen, etc.), and steroids (progesterone, deoxycorticosterone, etc.) [17]. In addition, Pdr5p seems to display a similar mechanism of substrate recognition and transport with that of human MDR protein. Thus, Pdr5p has been used to study human MDR mechanisms.

A number of compounds, such as FK506 (immunosuppressant) [7], flavonoids [8], protein kinase C effectors [9], and so on [10,11], have been reported to function as inhibitors against Pdr5p and MDR protein. However, other inhibitors are still desirable, since most

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of these compounds are not useful in medical applications because of their cytotoxicity.

We succeeded in the construction of a highly sensitive and specific assay system for Pdr5p inhibitor in *S. cerevisiae* [12]. We screened for microorganisms producing Pdr5p inhibitors using the original assay system. One of the isolates produced three kinds of enniatins (enniatin B, B1, and D) that were previously reported as ionophore antibiotics. In this paper, we described the inhibitory mechanisms of enniatins as Pdr5p inhibitors.

Materials and methods

Yeast strains, medium, and plasmids. *S. cerevisiae* KHW4 (*Mat α Δpdr5::LEU2*, *Δsnq2::HIS3*) and KHW3 (*Mat α Δsyr1/erg3::HIS3*, *Δpdr5::LEU2*, *Δsnq2::HIS3*) were derivatives of W303-1A. DKY1 (*Mat α leu2-3*, *-112*, *ura3-52*, *his3-Δ200*, *trp1-Δ901*, *lys2-801*, *suc2-Δ9*, *pdr-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 Iowa University [13]. Standard yeast media were used for growth of cells and drug resistance assays [16].

Chemicals. Cycloheximide and rhodamine 123 were purchased from Wako Pure Chemical Industries. Cerulenin and 4-nitroquinoline *N*-oxide (4-NQO) were purchased from Sigma–Aldrich Chemicals. FK506 was a gift from Fujisawa Pharmaceutical. Cycloheximide was dissolved in distilled water. Cerulenin, rhodamine 123, and FK506 were dissolved in ethanol. 4-NQO was dissolved in acetone.

Isolation of Fusarium sp. Y-53 and purification of enniatins from the isolate. By using our original assay system for Pdr5p inhibitors in yeast, strain Y-53 was obtained as a result of a search involving more than 7000 microorganisms isolated from soil [12]. According to 28S rDNA analysis, the strain was classified and named as *Fusarium* sp. Y-53. The medium for Pdr5p inhibitor production consisted of 20% potato extract supplemented with 1% glucose (pH 6.8). The strain Y-53 was cultured at 30 °C for 72 h (agitation rate, 350 rpm; aeration rate, 3 L/min) in a 3-L jar fermentor containing 2 L of the medium.

Pdr5p inhibitory assay. Pdr5p inhibitory assay was performed basically as described in our previous paper [12]. (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 [12]. (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 various concentrations of drugs (cycloheximide or cerulenin). 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 123 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 enniatin and then cultivated for an additional 2 h at 30 °C. Rhodamine 123 (final conc. = 10 μg/ml) was added and incubated for 30 min at 30 °C. After washing, the cells were resuspended with 1.0 ml phosphate-buffered saline (PBS) containing 2% glucose solution, and the fluorescence was visualized using a fluorescent microscope (Nicon Eclipse E-600, Japan).

Results

Purification of Pdr5p inhibitor from Fusarium sp. Y-53

The culture filtrate (1.5 L) was concentrated in vacuo and extracted with ethyl acetate. The crude extract was applied on a silica gel column (∅ 16×400 mm) and eluted with chloroform–methanol (95:5%, (v/v)). Active fractions were collected and then loaded on a CAPCELL PAC UG120 C-18 column (∅ 4.6×250 mm, SHISEIDO Fine Chemicals) using an HPLC system. The absorbed inhibitors were eluted stepwise as follows: (1) 70% acetonitrile–water (0–10 min), (2) 70–80% acetonitrile–water (10–20 min), and (3) 80% acetonitrile–water (20–30 min) at a flow rate of 0.8 ml/min. The monitoring of eluate was carried out at A_{210}. Accordingly, three Pdr5p inhibitors were obtained: peak A; total 3.5 mg (retention time:14.9 min), peak B; total 0.7 mg (retention time: 17.11 min), and peak C; total 2.1 mg (retention time: 18.18 min).

Chemical structure of enniatins

The chemical structures of the three purified Pdr5p inhibitors were determined by ^{1}H-NMR, ^{13}C-NMR, HMQC (INOVA-600 VARIAN), and MS spectrometry (JMS-700 spectrometer). Spectrum data of peak A were $\delta(^1\text{H})$ ($CDCl_3$) 5.13 (3H, d), 4.52 (3H, d), 3.13 (9H, s), 2.29 (3H, br m), 2.29 (3H br m), and 0.89–1.06 (36H m), $\delta(^{13}\text{C})$ ($CDCl_3$) 170.2, 169.1 75.7, 63.3, 33.4, 27.9, 29.9, 20.5, 19.2, 18.5, and 18.7 ppm *m/z* 639.4106 ($C_{33}H_{57}N_3O_9$ requires 639.4106). Based on a database search, the inhibitor was identified as enniatin B. In the same way, the other two inhibitors, peak B and peak C, were identified as enniatin D and B1, respectively (Fig. 1).

Inhibition of Pdr5p-mediated drug efflux by enniatins

Enniatin B (1–5 μg/ml, 0.8–7.8 μM) showed potent growth inhibition against the Pdr5p-overexpressing yeast cells when it was combined with cycloheximide or cerulenin, while enniatin B did not show any cytotoxic effects on either Pdr5p-deleted or -overexpressing cells without cycloheximide or cerulenin (Fig. 2). In addition, enniatin did not show growth inhibition for Snq2p-overexpressing yeast cells with 4-nitroquinoline N-oxide (4-NQO), a substrate of Snq2p (Fig. 2).

Enniatin B did not show growth inhibition up to 7.8 μM against Pdr5p-deleted cells (Figs. 3A and B) and Pdr5p-overexpressing cells in the absence of cycloheximide or cerulenin (Figs. 3C and D at the points of 0 μg/ml cycloheximide or cerulenin), while enniatin B showed growth inhibition in a dose-dependent manner against Pdr5p-overexpressing cells in the presence of cycloheximide or cerulenin (Figs. 3C and D). Enniatin