

Apoptosis-like Cell Death of *Saccharomyces cerevisiae* Induced by a Mannose-binding Antifungal Antibiotic, Pradimicin

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Pradimicin A (PRM), a mannose-binding antifungal antibiotic, induced the apoptosis-like cell death in *Saccharomyces cerevisiae*. The nuclear breakage and DNA fragmentation were observed in yeast cells by DAPI and TUNEL staining after the treatment with PRM. Accumulation of reactive oxygen species (ROS) was also detected in PRM-treated yeast cells by staining with dichlorodihydrofluorescein diacetate. PRM-induced cell death and the accumulation of ROS were prevented by pretreating the yeast cells with a radical scavenger, *N*-acetylcysteine. These results indicate that PRM induces the apoptosis-like cell death in yeast through the generation of ROS.

Pradimicin, a benzo[*a*]naphthacenequinone antibiotic, was first isolated from *Actinomadura hibisca* in 1988, and a number of congeners and derivatives have been reported¹). It exhibits potent and highly selective antifungal activity against *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*²). In addition, it inhibits the infection of human immunodeficiency virus to T cells³) or enhances the syncytium formation of human parainfluenza virus-infected HeLa cells⁴). It also inhibits the osteoclast formation from hematopoietic precursors⁵). These biological activities of pradimicin appear to be caused by its binding to the cell surface mannose-rich oligosaccharides.

Since most mammalian cells do not usually express mannose-rich oligosaccharides, they are not sensitive to pradimicin. However, after the cultivation in the presence of 1-deoxymannojirimycin (DMJ), the cells express high mannose type oligosaccharides and therefore become sensitive to pradimicin. In such DMJ-preincubated cells, pradimicin induces apoptosis, accompanied by the DNA fragmentation, elevation of intracellular Ca²⁺ level and generation of reactive oxygen species⁶⁻⁸).

Pradimicin binds to the cell surface mannan and induces

a rapid cell death in *C. albicans*⁹⁻¹²), but its mode of action remains unknown. In this paper, we describe the characteristic morphological changes of apoptosis in *Saccharomyces cerevisiae* caused by pradimicin. This is the first identification that an antifungal antibiotic induces apoptosis-like cell death in yeast.

Materials and Methods

Compound

Pradimicin A was isolated from the culture broth of actinomycete sp. strain TP-A0016¹³).

Microorganism and Growth Conditions

The yeast *S. cerevisiae* 953: ATCC52052 (MAT α *his4-713*, *leu2-3*, *leu2-112*, *ura3-52*, *met2-1*, *lys2*) was used. This organism was maintained on YPD agar consisting of 1% yeast extract (Difco), 2% Bacto peptone (Difco), 2% glucose and 1.5% agar. The yeast cells were subcultured in YPD medium in a 250 ml flask and incubated on a mechanical shaker at 26°C to the mid-exponential phase.

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PRM Treatment and *N*-Acetylcysteine (NAC) Pretreatment

Exponential-phase cells pre-grown in YPD medium were harvested, washed twice with phosphate buffer saline (PBS), and then suspended (10^6 cells/ml) in SD medium consisting of 0.67% yeast nitrogen base (Difco) and 2% glucose with supplements, 20 μ g/ml of methionine, histidine, leucine, lysine and uracil. The treatments were carried out for 2 hours at 26°C in the cultured medium containing 0 or 20 μ g/ml of pradimicin A (PRM). *N*-Acetylcysteine (NAC) treatment (0~5 mM) was carried out with the exponential-phase cells for 2 hours at 26°C in the same medium as described above, and then PRM was added to the cultured medium. After the treatment, a portion of yeast cells were inoculated on YPD plates containing the same concentrations of NAC (0~5 mM) and incubated for 2 days at 26°C. The number of colonies was counted up to 300 in average and the percentage viability was determined by dividing the number of colonies from the PRM-treated culture with that from the untreated culture.

Detection of DNA Fragmentation: Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick and Labeling (TUNEL) Staining

DNA strand breaks were detected by TUNEL using 'In situ apoptosis detection kit' (Takara Bio Inc.). This reagent labels the free 3'-OH termini with FITC-labelled deoxyuridine triphosphate (dUTP), which is visible on a fluorescence microscopy. Yeast cells were fixed with 4% formaldehyde, digested with Zymolyase (Seikagaku Co.) and applied on a silicon-coated slide¹⁴). The slide was rinsed with PBS, incubated in permeabilisation buffer for 2 minutes on ice, rinsed with PBS. Then it was incubated with 50 μ l TUNEL reaction mixture (45 μ l of Labelling safe buffer and 5 μ l of TdT Enzyme containing terminal deoxynucleotidyl transferase and FITC dUTP) for 1 hour at 37°C, and then rinsed three times with PBS. Observations were carried out using an Olympus model BHS-RFK, fluorescence microscope equipped with an USH-102D mercury lamp, excitation filter BP545 and DM580 (O590).

Nuclear Staining and Reactive Oxygen Species (ROS) Staining

Nuclear staining: After treating with PRM, yeast cells were washed with PBS, resuspended in 70% (v/v) ethanol, incubated with 1 μ g/ml diamidinophenylindole diacetate (DAPI: Sigma) in PBS for 30 minutes and applied on slides.

ROS staining: After treating with PRM, yeast cells were washed, incubated with 10 μ g/ml of dichlorodihydrofluorescein diacetate (DCF-DA: Sigma) for 2 hours¹⁵) and applied on slides. The slides were observed on an Olympus model BHS-RFK, fluorescence microscope.

Results and Discussion

No apoptosis-related proteins in metazoans are found in the BLAST database search of *S. cerevisiae* genome. The expression of metazoan proapoptotic genes (Bak, Bax, Ced-4) in yeast cells, however, causes cell death with apoptotic morphology and the Bax-induced yeast cell death is protected by the expression of Bcl-2, an apoptosis suppressor¹⁶⁻¹⁹). In addition, a point mutation of *CDC48* (*cdc48*^{S565G}) in *S. cerevisiae*, which codes a protein responsible for the regulation of cell division cycle, induces the cell death with typical morphological and molecular markers of apoptosis¹⁴). It is, therefore, proposed that the basic machinery of apoptosis is present and functional also in the unicellular organism, yeast.

Chromatin condensation and fragmentation is a typical marker of apoptosis. DAPI-staining of yeast cells which were treated with 20 μ g/ml pradimicin A (PRM, Fig. 1) indicated the randomly distributed nuclear fragments in the cells (Fig. 2). Apoptotic DNA cleavage produces free 3'-OH termini, which can be detected by labeling with fluorescent-labeled nucleotides catalyzed by terminal deoxynucleotidyl transferase, the TUNEL method. The PRM-treated yeast cells have an intensive yellow-orange stain with the TUNEL assay, indicating a strong fragmentation. The orange stain is probably due to the

Fig. 1. Structure of pradimicin A.

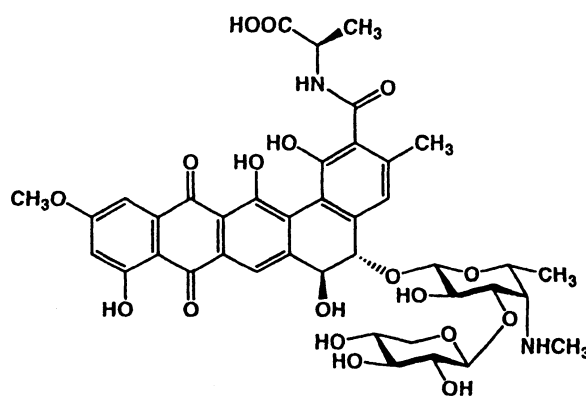
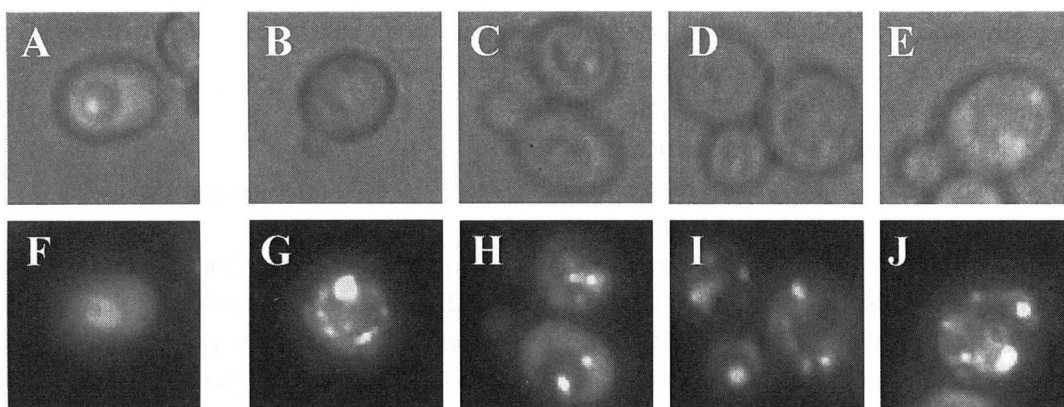


Fig. 2. PRM-treated *S. cerevisiae* after staining of nuclei with DAPI.

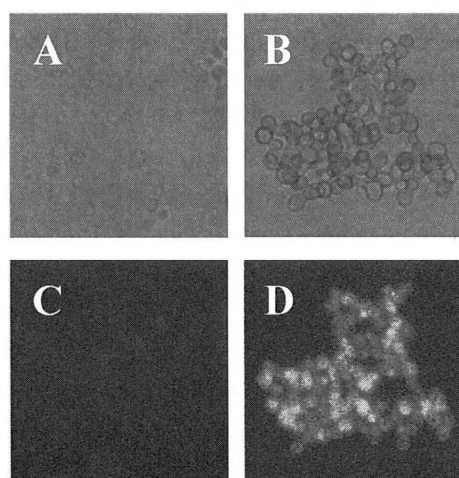


A/F: control, B/G, C/H, D/I and E/J: treated with 20 $\mu\text{g/ml}$ of PRM for 2 hrs.
A-E and F-J were shown as bright and fluorescent field, respectively.
Yeast cells were stained by incubating with diamidinophenylindole acetate for 30 min.

absorption of the FITC-fluorescence by PRM bound to the cell surface. More than 90% of the PRM-treated cells were stained by TUNEL whereas the control cells were unstained (Fig. 3). These cellular changes are the markers for apoptosis in metazoans. Additionally, most of the PRM-treated cells were not stained with propidium iodide, suggesting that the manner of this cell death is not necrosis (results not shown). In the study of morphological changes in *C. albicans* induced by pradimicin using electron microscopy, BMY-28864, a pradimicin derivative, was shown to induce cell membrane invaginations together with cell membrane detachment from the cell wall, nuclear membrane fragmentation and mitochondrial aberration in a short period¹²). These phenomena were also observed in the yeast mutant in *CDC48* which showed typical markers of apoptosis²⁰).

Reactive oxygen species (ROS) are another markers for apoptosis. The accumulation of ROS was observed in the apoptosis of yeast mutant in *CDC48*²¹). The radicals were necessary to induce the apoptotic morphology because glutathione prevented the apoptotic death. More than 90% of yeast cells, which were treated with 20 $\mu\text{g/ml}$ PRM for 2 hours, were stained with DCF-DA, indicating a generation of ROS. Most of the control cells were not stained (Fig. 4). To test the involvement of oxygen radicals, the effect of a radical scavenger, *N*-acetylcysteine (NAC)^{22,23}) on PRM-induced cell death was examined. The viability of the cells, which were incubated with 20 $\mu\text{g/ml}$ PRM without

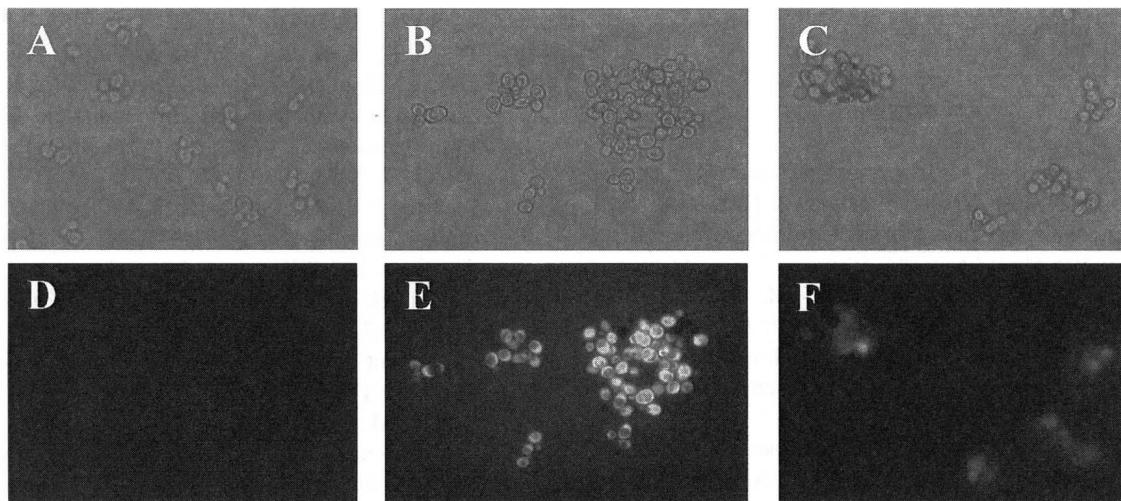
Fig. 3. DNA fragmentation of PRM-treated *S. cerevisiae* detected by TUNEL.



A/C: control, B/D: treated with 20 $\mu\text{g/ml}$ of PRM for 2 hrs.
A and B, and C and D were shown as bright and fluorescent field, respectively. Yeast cells were stained by TUNEL.

NAC pretreatment, was approximately 2%. The viability increased with the increase of NAC concentration and over 80% of the cells survived by the pretreatment with 5 mM NAC (Fig. 5). This result was consistent with the

Fig. 4. ROS production in PRM-treated *S. cerevisiae* after staining with 2,7-dihydrodichlorofluorescein.



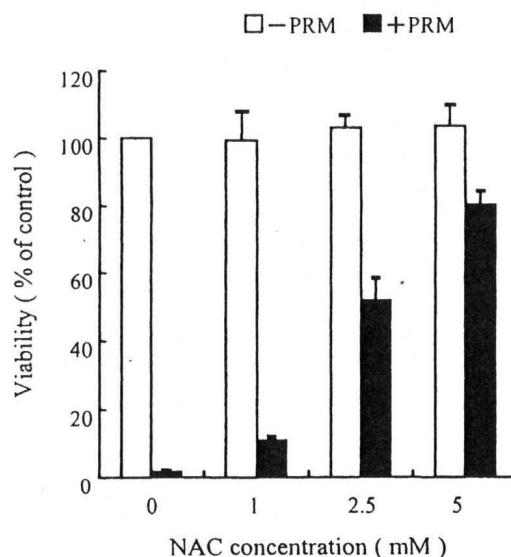
A/D: control, B/E and C/F: treated with 20 µg/ml of PRM for 2 hrs,
C/F: pretreated with 5 mM *N*-acetylcysteine before PRM-treatment.
A-C and D-F were shown as bright and fluorescent field, respectively.
Yeast cells were stained by incubating with 10 µg/ml of 2,7-dihydrodichlorofluorescein for 2 hrs.

observation that less than 10% of these NAC-pretreated yeast cells were stained with DCF-DA after the treatment with PRM (Fig. 4). The chemical stability of PRM during the incubation with NAC was confirmed by LC/MS and the antifungal activity of PRM was fully retained after the incubation with NAC (results not shown).

In this study, it was shown that DNA fragmentation, nuclear breakage and accumulation of ROS occurred in yeast cells by the treatment with PRM, and that the cell death and ROS accumulation was canceled by pre-incubation with NAC, an oxygen radical scavenger. These observations indicate that PRM induces the ROS formation and the ROS induces the cell death in yeast and that this cell death corresponds to the apoptosis in metazoan systems. This is the first confirmation of the apoptotic cell death of yeast induced by an antifungal antibiotic.

Pradimicin recognizes mannosides in the similar manner as lectin and binds to the yeast cell surface in the presence of calcium ions¹¹. The binding is essential for pradimicin to exert its antifungal action, because the antifungal activity is antagonized by mannose or mannosides¹¹. Concanavalin A is a lectin that recognizes mannosides²⁴ as well as pradimicin, but it does not show antifungal activity to *S. cerevisiae* (unpublished result). Therefore we speculate that pradimicin binds to a specific manno-protein receptor on

Fig. 5. Effect of *N*-acetylcysteine on PRM-induced apoptotic cell death in *S. cerevisiae*.



Yeast cells were preincubated with various concentrations of NAC for 2 hrs and treated with 20 µg/ml of PRM for 2 hrs. Viability was determined by counting the growing colonies on YPD plates.

the cell membrane in yeast in addition to the non-specific binding to the cell surface mannan layer. We have isolated a number of classes of pradimicin-resistant mutants of *S. cerevisiae*. The genetic analysis of the mutants will be discussed in a forthcoming paper.

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