A Novel Method of Screening Cell-Cycle Blockers as Candidates for Anti-Tumor Reagents Using Yeast as a Screening Tool

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The mechanisms of eukaryotic cell-cycle regulation are closely linked to cellular tumorigenesis. Compounds that affect the cell cycle are good candidates for developing anti-tumor drugs. We developed a screening method for cell-cycle blockers using a Saccharomyces cerevisiae cdc2-1 rad9Δ strain that can detect the activity of substances by cell growth. We performed screening on culture broth of various microbes, and identified five compounds, borrelidin, mycophenolic acid, UCS15A, copiamycin analog, and fredericamycin A, that were known to possess anti-tumor activity. These results indicate that this screening method is effective as a first-screening system for anti-tumor agents.

Key words: cell-cycle blocker; checkpoint control; screening method for anti-tumor drugs; Saccharomyces cerevisiae

Most eukaryotic cells proceed through an ordered series of events constituting the cell cycle. Regulation of the cell cycle, especially the cell-cycle checkpoint control system, which ensures the intactness of chromosomes, is critical to the normal development of multicellular organisms. Loss of control ultimately leads to cancer. The budding yeast Saccharomyces cerevisiae has been especially useful in the isolation of mutants. Genetic- and molecular genetic studies using the budding yeast have greatly contributed to understanding of cell-cycle regulation in eukaryotes. In addition to its characteristics as a model eukaryotic cell in molecular- and cell-biological research, the yeast has technical advantages, such including simple growth conditions, rapid cell division, and the development of a wealth of genetic tools for analysis of biological functions. These characteristics have expanded the application of the yeast as screening tool to the field of drug discovery.1) We conceived of a possibility, that cell-cycle blockers or DNA-damage-checkpoint activators can be screened using a yeast checkpoint mutant strain, and we developed a novel screening method that can detect the activity of substances by cell growth. In this report, we describe the method and the results of screening of culture fluids of actinomycetes and fungi to show that our method can effectively identify cell-cycle blockers.

The RAD9 gene is the first one to be identified as figuring in cell-cycle checkpoint control in S. cerevisiae.2) RAD9 null mutants are viable but exhibit sensitivity to X-ray and UV irradiation, fail to arrest the cell cycle in response to DNA damage, and are prone to chromosomal instability. We disrupted the RAD9 gene in a cdc2-13) mutant background by replacing the RAD9 ORF with the URA3 gene by PCR-directed one-step gene disruption (WCTR312A, MATa ade2 his3 trp1 ura3 rad9Δ::URA3 cdc2-1). CDC2 encodes a catalytic subunit of DNA polymerase δ, required for chromosomal DNA replication during mitosis and meiosis. cdc2-1 mutant cells stop DNA replication at the restrictive temperature, 37 °C, and arrest at the S phase through the functions of DNA-damage checkpoint control. cdc2-1 rad9Δ double mutant cells rapidly lose their viability at 37 °C because they lack the RAD9 product, but they survive in the presence of the cell-cycle blocking agent thiabendazole (TBZ), because TBZ induces mitotic spindle-checkpoint control to arrest the cell cycle of the mutant at the M phase. The arrested cells can override the arrest by a mechanism called adaptation even if they are unable to repair the damage that evoked the checkpoint response,4) or by a mechanism called recovery, after they repair the damage. Thus, cdc2-1 rad9Δ double mutant cells transiently treated at the restrictive temperature (37 °C) in the presence of a cell-cycle blocker can continue the cell cycle when they are shift back to a permissive temperature (25 °C), while in the absence of the blocker they can not.

To test whether this mutant strain can be used as an assay system to detect cell-cycle blocking agents, we spread 1 × 107 log-phase cells of WCTR312A grown at 25 °C on a YPD (2% glucose, 2% peptone, 1% yeast extract, 2% agar) agar plate, put them on 5-mm diameter paper discs soaked with 50 or 30 μl of 2 μl of hydroxyurea (HU) or 10 mg/ml TBZ in dimethyl sulfoxide (DMSO), and incubated this at 25 °C for 2 d or at 37 °C for 12 h,
and then shifted to 25°C for a further 36 h. As shown in Fig. 1A, WCTR312A cells grew as a lawn at 25°C with a weak growth inhibition zone around 50 µl HU and TBZ discs. On the other hand, most of the cells incubated at 37°C for 12 h did not grow, except around the discs containing HU or TBZ. The size of the growth zone correlated with the dose of the drug, indicating that WCTR312A is suitable for bioassay for cell-cycle blockers. Next we tested to determine whether this assay can be used for high-throughput screening, by embedding WCTR312A cells in an agar plate and spotted with 1 µl of serial dilutions of HU or TBZ. As shown in Fig. 1B, clear growth zones were detectable where HU or TBZ was spotted, but no growth zone was detectable with the vehicles (H2O and DMSO respectively). In order to evaluate this assay system, we prepared a culture supernatant of about 12,000 strains of actinomycetes and fungi within a culture collection from Research Institute of Life Science, Snow Brand Milk Products, and tested their ability to support the growth of 37°C-treated WCTR312A cells. These culture supernatants were directly spotted twice on YPD plates that were embedded with WCTR312A cells using a 48-pin applicator (1 to 2 µl/spot). The plates were incubated at 37°C for 12 h and then shifted to 25°C for another 36 h. HU and medium for actinomycetes (2% glucose, 1% soluble starch, 0.1% meat extract, 0.4% dried yeast, 2.5% soybean flour, 0.2% NaCl, and 0.005% K2HPO4 pH 6.7) or fungi (2% glucose, 1% glycerol, 1% lactose, 1% sucrose, 3% soybean flour, 0.8% polypeptone, 0.2% sodium nitrate, and 0.1% MgSO4·7H2O pH 6.8) were used as positive- and negative controls. Typical results of this assay are shown in Fig. 1C. For the candidate strains whose culture broth supported the growth of WCTR312A cells at 37°C, we repeated this assay 3 times with independently prepared culture supernatants, and found that the culture broth of five strains termed B1 to B5 reproducibly showed potent activity. The active compound was then purified from a culture broth of these strains by ethyl-acetate- (for B1, B2, B3, and B5) or BuOH extraction (for B4), silica gel column chromatography (B1 and B5: CHCl3:CH3OH = 10:1, B2: CHCl3:CH3OH = 50:1 to 10:1, B3: CHCl3:CH3OH = 50:1, B4: CHCl3:CH3OH = 1:1), and HPLC (ODS column, B1: 85% MeOH, B2: 80% MeOH–0.1% trifluoroacetic acid (TFA), B3: 70%MeOH–0.1% TFA, B4: CH3CN-0.1 M NaHPO4, B5: 50%CH3CN). The UV, 1H NMR, and 13C NMR spectra indicated that these compounds were identical to borrelidin 5) (B1), micophenolic acid 6) (B2), UCS15A 7) (B3), copiamycin analog 8) (B4), and fredericamycin A 9) (B5) (Table 1, Fig. 2A). B3 and B4 consisted of 4 and 2 related compounds (B3A to B3D, and B4A and B4B, respectively). While we could

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Fig. 1. Effects of Hydroxyurea (HU) and Thiabendazole (TBZ) on the Growth of WCTR312A Cells.

A, 1 × 10^7 log-phase cells of WCTR312A were spread on a YPD agar plate, and 5-mm diameter paper discs soaked with 50- or 30 µl of 2 mM HU or 10 mg/ml TBZ in DMSO were placed on the plate. The plates were incubated at 25°C for 2 d (left panel) or at 37°C for 12 h, and then shifted to 25°C for another 36 h (right panel). B, 1 × 10^7 log-phase cells of WCTR312A were embedded in a YPD agar plate, and 1 µl of 2-fold serial dilutions of HU (2 mM) or TBZ (10 mg/ml) and their vehicle, H2O and DMSO respectively, were spotted on the upper and lower lines. The plates were incubated at 37°C for 12 h and then shifted to 25°C for another 36 h. C, Typical results of screening with the culture supernatant of actinomycetes strains. The samples showing clear growth as indicated by arrowheads, were tested for reproducibility. N and P are negative and positive controls respectively.
Table 1. Results of Screening

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Sourcea)</th>
<th>Substance</th>
<th>IC50 (μg/ml)</th>
<th>Effects on Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>SNA-24891</td>
<td>borrelidin</td>
<td>2</td>
<td>G1 arrest, CDK inhibition</td>
</tr>
<tr>
<td>B2</td>
<td>SNF-5362</td>
<td>micophenolic acid</td>
<td>8</td>
<td>slow growth</td>
</tr>
<tr>
<td>B3</td>
<td>SNA-23252</td>
<td>UCS15A</td>
<td>3</td>
<td>cytokinesis defect</td>
</tr>
<tr>
<td>B4</td>
<td>SNA-22058</td>
<td>copiamycin analog</td>
<td>8</td>
<td>G1 delay</td>
</tr>
<tr>
<td>B5</td>
<td>SNA-16232</td>
<td>fredericamycin A</td>
<td>0.013</td>
<td>G1 arrest, agglutination of mitochondria</td>
</tr>
</tbody>
</table>

*a) The SNA- and SNF strains were actinomycetes and fungal strains respectively of the culture collection of Research Institute of Life Science, Snow Brand Milk Products.

Fig. 2. Chemical Structures and Effects of Drugs on the Growth of W303-MLC30 Cells.

A. Chemical structures of drugs. B. Effects of the drugs on the growth of W303-MLC30 cells. Liquid cultures of W303-MLC30 (2 × 10⁶ cells/ml) were added with each drug (closed circle) or the vehicle (open circle, DMSO for UCS15A and ethanol for the other drugs) and incubated with shaking at 28°C, except for UCS15A (37°C). In the culture of borrelidin, YPD medium was adjusted to pH 4.5. Drug concentrations used were; borrelidin, 4μg/ml; micophenolic acid, 20μg/ml; UCS15A, 5μg/ml; copiamycin analog, 20μg/ml; and fredericamycin A, 0.1μg/ml.
identify the structures of B3C and B4A, the structure of the others could not be determined due to small quantity. Although these drugs have been reported to possess anti-fungal and/or anti-tumor activities, the effects of the drugs on yeast growth have not been examined. The purified drug was dissolved in ethanol or DMSO at 5 mg/ml and used in further experiments using a drug sensitive strain W303-MLC30 (5 mg/ml and 112 trp1-1 his3-11 ade2-1 can1-100 yrs1Δ::HIS3 yrr1Δ::TRPI pdr1Δ::hisG-URS3-hisG pdr3Δ::hisG-URS3- hisG). W303-MLC30 is a strain carrying deletion of multi-drug resistance genes, and showed 2–10 times higher sensitivity to the drug than the parental strain, W303-1A. The half-inhibitory concentration (IC50) of each compound on yeast cell growth estimated by fold increase of cell numbers during a 10-h incubation was as indicated in Table 1. Because borreridin and UCS15A showed higher growth inhibitory activity at lower pH (pH 4.5) and at higher growth temperature (37°C), respectively, IC50 value determination and further analysis of these compounds were carried out under these conditions. The concentrations of the compounds for over 90% growth inhibition were as follows: borreridin, 4 μg/ml; micophenoric acid, 20 μg/ml; UCS15A, 5 μg/ml; copiamycin analog, 20 μg/ml; and fredericamycin A, 0.1 μg/ml. The growth of W303-MLC30 cells in the presence of the drugs at concentrations of over 90% inhibition is shown in Fig. 2B. To assess the effects of these compounds on cell-cycle progression, we examined the DNA content of the cells by fluorescence-activated cell sorting (FACS) analysis. Because W303-MLC30 cells stop growth within 6 h of incubation with these drugs (Fig. 2), approximately 1 x 107 cells treated with each drug for 6 h were withdrawn from these cultures and processed for propidium iodide staining. For each preparation, 20,000 events were analyzed for DNA content using an Epics Elite flow cytometer. The borrelidin- and fredericamycin A treated cells showed G1 arrest as described previously. The ratio of the drug-treated cells containing 1C- or 2C DNA was 85:15, while that of propidium iodide treated cells was 40:60. The copiamycin analog-treated cells showed G1 delay (ration of 1C- and 2C DNA: 65:35), and the UCS15A-treated cells showed a defect in cytokinesis to accumulate the cells with 4C-DNA content, while the micophenolic acid-treated cells were indistinguishable from the vehicle treated cells (data not shown, to be described elsewhere). These results strongly suggest that our screening method can be a powerful tool to identify cell-cycle blockers. Five compounds that were identified in this study are known to possess anti-tumor activity. In addition, recently we isolated bisabolane sesquiterpenoid endoperoxide, 3,6-epidioxy-1,10-bisaboladiene (EDBD) from edible wild plant Cacalia delphinifolia which induces apoptosis in human chronic myelogenous leukemia K562 and human prostate carcinoma LNCaP cell lines using this screening method. Our procedure is based on the positive effect of the drugs on cell growth, and provides the advantage of eliminating the possibility of isolating cytotoxic compounds. The screening procedure described here is expected to be a powerful tool for the first-screening system to identify anti-tumor agents.

References