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A novel yeast cell-based screen identifies flavone as a tankyrase inhibitor

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ABSTRACT

The telomere-associated protein tankyrase 1 is a poly(ADP-ribose) polymerase and is considered to be a promising target for cancer therapy, especially for BRCA-associated cancers. However, an efficient assay system for inhibitor screening has not been established, mainly due to the difficulty of efficient preparation of the enzyme and its substrate. Here, we report a cell-based assay system for detecting inhibitory activity against tankyrase 1. We found that overexpression of the human tankyrase 1 gene causes a growth defect in the fission yeast *Schizosaccharomyces pombe*. Chemicals that restore the growth defect phenotype can be identified as potential tankyrase 1 inhibitors. We performed a high-throughput screen using this system, and identified flavone as a compound that restores the growth of yeast cells overexpressing tankyrase 1. Indeed, flavone inhibited poly(ADP-ribosyl)ation of proteins caused by overexpression of tankyrase 1 in yeast cells. This system allows rapid identification of inhibitory activity against tankyrase 1 and is amenable to high-throughput screening using robotics.

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1. Introduction

Poly(ADP-ribose) polymerases (PARPs) catalyze the covalent attachment of poly(ADP-ribose) (PAR) polymers to a protein substrate. PAR polymers are assembled from monomers of the enzyme's substrate, nicotinamide adenine dinucleotide (NAD^+). The PARP family includes at least 17 family members, which are involved in a wide range of cellular processes [1]. One family member, tankyrase 1 (TRF1-interacting ankyrin-related ADP-ribose polymerase 1) was originally identified in a yeast two-hybrid screen as a protein binding to telomeric repeat binding factor, TRF1 [2,3]. Telomeres are the unique DNA–protein complexes at the eukaryotic chromosome ends. TRF1 binds to the telomeric DNA and negatively regulates telomere length [4]. Tankyrase 1 poly(ADP-ribosyl)ates TRF1, releasing TRF1 from telomere DNA and stimulating ubiquitin-mediated proteolysis of TRF1 [5]. Thus, tankyrase 1 is a positive regulator of telomere length. In human, germ cells and most cancer cells exhibit a high level of telomerase activity that maintains telomere length, allowing these cells to divide indefinitely [6,7]. Therefore, telomerase is one of the attractive targets for cancer therapy. To date, several telomerase inhibitors have been developed and reported (e.g., GRN163L and MST-312) [8,9]. In this context, tankyrase 1, another positive regulator of telomeres, is also a potential target for telomere-directed anticancer therapeutics. A recent report showed that inhibition of tankyrase 1 was lethal only when combined with BRCA (breast cancer associated) deficiency. Loss of BRCA function is associated with breast cancer as well as malignancies of the ovaries, pancreas, and prostate gland [10]. This synthetic lethal interaction is an attractive therapeutic approach because treatment with a single agent that targeted tankyrase 1 would be expected to specifically kill cancer cells, but not most normal cells. Prior to the proposal of targeting tankyrase 1 as a therapeutic strategy for BRCA-associated cancer, inhibition of the DNA repair enzyme PARP1 was known to show selective toxicity against BRCA-deficient cells [11,12]. However, due to patients' refractoriness to PARP1 inhibitors or acquisition of drug resistance, it became important to identify alternative therapeutic targets for BRCA-associated cancers. Therefore, the development of tankyrase 1 inhibitors is a promising approach to anticancer therapeutics. Recently, XAV939 was identified as a tankyrase inhibitor during screening for a small-molecule

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inhibitor of the Wnt/β-catenin pathway [13]. In the Wnt/β-catenin pathway, axin, a component of the destruction complex that regulates β-catenin, is degraded by poly(ADP-ribosyl)ation catalyzed by tankyrase 1 and its isoform, tankyrase 2. So far, there has been no other report of screening for tankyrase 1 inhibitors, probably due to the difficulty of efficiently preparing active tankyrase 1. Here, we describe an efficient system for screening tankyrase 1 inhibitors, amenable to a high-throughput method, using fission yeast strain overexpressing human tankyrase 1 (TNKS1). A yeast cell-based phenotypic drug screening system has advantages over *in vitro* assay system, because cell-permeability and toxicity of chemical compounds can be simultaneously tested during screening. *Schizosaccharomyces pombe* is an excellent model system: genetic manipulation is easy; it is cost-effective; and the cells grow rapidly, making possible rapid and high-throughput drug screening. Moreover, *S. pombe* has no poly(ADP-ribose) polymerase homolog. Thus, this yeast can be used as a "living test tube" to detect the enzymatic activity of the ectopically expressed PARPs. Taking advantage of this approach, we identified flavone as a tankyrase 1 inhibitor.

2. Materials and methods

2.1. Reagents

Tested compounds were supplied from the RIKEN Natural Products Depository (NPDepo). WST-1 and 1-methoxy-PMS were obtained from Dojindo, and both reagents were solubilized in 20 mM Hepes (pH 7.4). These compounds were added to the yeast culture at final concentrations of 0.25 mM for WST-1 and 0.01 mM for 1-methoxy-PMS. Flavone, 3-aminobenzamide (3AB), a mouse monoclonal anti-tubulin antibody (B5-1-2), and a mouse monoclonal anti-FLAG antibody (M2) were purchased from Sigma. Mouse monoclonal anti-PAR antibody (clone 10HA) was purchased from Trevigen, and rabbit polyclonal anti-tankyrase (H-350) antibody was purchased from Santa Cruz Biotechnology. The horseradish peroxidase (HRP)-linked anti-mouse IgG and anti-rabbit IgG and the FITC-conjugated anti-mouse IgG were obtained from GE Healthcare. The VECTASHIELD Mounting Medium was purchased from Vector Lab.

2.2. Introduction of the human TNKS1 into the fission yeast cells

The human TNKS1 gene was cloned as Gateway entry clones by recombining the PCR-amplified open reading frame (ORF) of human cDNAs as reported [14]. The TNKS1 entry clones were produced as an N-type clone, with an intrinsic STOP codon at the end of the ORF, and as an F-type clone with no STOP codon, for producing a C-terminal tagged protein. Sequence analysis revealed that the PCR-amplified TNKS1 gene contained three nucleotide mutations (C248A, A3002G, and G3098A). The PARP-dead mutant TNKS1 carrying a single amino acid change (H1184A) was constructed as follows: the point mutation in the PARP domain was obtained by PCR amplification of the Gateway entry plasmid containing TNKS1 using the primers tank1_H1184A_Fwd 5′-GAGCGCATGTTGTTTGCTGGTTCTCCT TTCATTAATG-3′ and tank1_H1184A_Rev 5′-CATTAATGAAAGGAGA ACCAGCAAACAACATGCGCTC-3′. Both types of the wild-type (WT) and mutant TNKS1 genes were transferred to the fission yeast expression vector pDUAL-FFH1c by recombination, in order to produce the native-type TNKS1 protein (N-type) or the C-terminally FLAG–FLAG–His_6-tagged TNKS1 protein (F-type), respectively, under the control of the thiamine-regulatable *nmt1* promoter [15,16]. Yeast genetic manipulation was performed as described [17]. In order to elevate the drug sensitivity of the fission yeast strains, we deleted the *pmd1* and *bfr1* genes, which encode ABC transporters responsible for an efflux of various antibiotics [18,19]. The *pmd1::ura4* fragment [18] was PCR-amplified using primers that flanked the *pmd1* gene (SN6F1, 5′-TTCGTTTTATCAATTCCATT-3′; SN6R1, 5′-AATAAAACACAAACCTGACT-3′) [20] and was inserted into the *pmd1* locus of strain JY878 (*h^{90} ade6-M216 leu1-32 ura4-D18*), producing the strain YY278 (*h^{90} ade6-M216 leu1-32 ura4-D18 pmd1::ura4*). YY278 was crossed with YY269 (*h^{-} ade6-M210 leu1-32 ura4-D18 bfr1::ura4*), which was constructed in Ref. [19]. The resultant diploid cells were sporulated and subjected to dissection. One of the progeny, YY299 (*h^{90} ade6-M216 leu1-32 ura4-D18 pmd1::ura4 bfr1::ura4*), was chosen for use as the drug-sensitive host strain. The gene disruption was verified by uracil prototrophy and PCR. To produce the TNKS1 overexpression strains, we introduced the N-type and F-type TNKS1 genes, with or without catalytic mutations, into the *leu1* locus of YY299. Although the PCR-amplified TNKS1 gene contains three nucleotide mutations (C248A, A3002G, and G3098A), resulting in amino acid alterations (Pro83Gln, Glu1001Gly, and Met1266Ile), we concluded that these changes cause no adverse effect on the enzyme activity of tankyrase 1, since overexpression of TNKS1 showed sufficient enzyme activity in yeast (see below).

2.3. High-throughput drug screening using a WST-1 reagent

We used WST-1 to quantify yeast cell growth based on mitochondrial dehydrogenase activity, which cleaves the tetrazolium salt WST-1 to produce the formazan dye. Fission yeast cells pre-grown on synthetic dextrose (SD) solid medium at 30 °C for 2–3 days were subsequently grown for 24 h with vigorous shaking in minimal medium (MM) containing 10% (v/v) of SD liquid media, in order to supply a small amount of thiamine. This pre-culture was then 200-fold diluted in MM media and grown at 30 °C for 18 h. Finally, the prepared WST-1 mixture was added to the yeast culture, and absorbance of the formazan product was measured after incubation for more than 3 h (absorption wavelength, 450 nm; reference wavelength, 650 nm). For the primary high-throughput drug screening, yeast cells were cultured in 20 μL of MM media containing chemical compounds from the NPDepo library at concentrations of 10, 5, 2.5, and 1.25 μg/mL using 384-well plates (see Supplementary Fig. 1); all cultures were dispensed using a Biomek NX automation instrument. The WST-1 mixture was added to the yeast culture using a Biotec mini-Gene LD-01 single-line dispenser device. Thorough vortexing of the 384-well microplates was performed using a Biotec Bio-mixer. We selected the compounds whose *Z* scores of absorbance calculated every 88 chemical compounds were over 10 as hits. For the secondary assay, yeast cells were cultured in 50 μL of MM media with varying concentrations of flavone or 3AB using 96-well plates, and WST-1 mixture was added to the culture.

2.4. Immunoblotting

Cells grown in MM media with or without drugs were harvested after 18–22 h. Lysates were prepared as described [21] and analyzed by SDS–PAGE and Western blotting using the anti-PAR antibody (1:1000), the anti-tankyrase antibody (1:1000), the anti-tubulin antibody (1:5000), the HRP-linked anti-mouse antibody (1:10,000), or the HRP-linked anti-rabbit antibody (1:10,000).

2.5. Immunofluorescence staining

Indirect immunofluorescence staining was performed as described [22]. Briefly, HeLa I.2.11 cells were transfected with pLPC/FN-tankyrase-1 vector [3] using a standard electroporation method. After incubation for 18–21 h with test compounds at 37 °C, cells were fixed with 2% paraformaldehyde/phosphate-buffered saline (PBS) and permeabilized with 0.5% Nonidet P-40/PBS. Cells

were blocked in PBS containing 1% bovine serum albumin, and incubated with mouse anti-FLAG antibody (2 μg/mL). Next, the cells were washed and further incubated with FITC-conjugated sheep anti-mouse immunoglobulin (1:25). Cellular DNA was stained with 4,6-diamino-2-phenylindole (DAPI) using the VECTA-SHIELD Mounting Medium. Images were collected using an Olympus IX-71 microscope with a DP70 digital camera.

3. Results

3.1. Construction of fission yeast strains that overexpress human tankyrase 1

To test the effects of TNKS1 gene overexpression on the cell growth of *S. pombe*, we introduced PCR-amplified human TNKS1 genes into a drug-sensitive strain lacking both *pmd1* and *bfr1* [18,19]. The native form of the TNKS1 gene, containing the intrinsic termination codon (N-type), and the C-terminally FLAG/His_6-tagged form (F-type) were integrated into the *leu1* locus. The genes were under control of the thiamine-regulatable *nmt1* promoter: expressed on MM media lacking thiamine, but repressed on SD media containing thiamine. Expression of both the N-type and F-type TNKS1 genes caused growth retardation in fission yeast cells on MM, but not under uninduced conditions on SD (Fig. 1A). Overexpression of the F-type TNKS1 gene caused a more severe growth defect than that of N-type, because of the higher enzymatic activity of the F-type tankyrase 1 protein (see below, Fig. 1B). It seems possible that C-terminal tagging has some role in supporting correct folding in the fission yeast cells. In contrast to the growth defect of the WT TNKS1 overexpression strains, overexpression of the PARP-dead mutant (H1184A) failed to induce growth arrest (Fig. 1A). Furthermore, immunoblotting analysis with the anti-PAR antibody showed that poly(ADP-ribosyl)ation of proteins occurred in *S. pombe* cells overexpressing WT TNKS1 (both N-type and F-type), but not in those overexpressing the catalytically dead TNKS1 (Fig. 1B). In mammalian cells, known targets of tankyrase 1 are TRF1 and axin, neither of which are conserved in fission yeast [5,13]. Since tankyrase 1 is auto-poly(ADP-ribosyl)ated, the major poly(ADP-ribosyl)ated protein with a molecular mass of around 140 kDa could be the tankyrase 1 protein itself overexpressed in fission yeast (see Fig. 1B, lane 3). Other poly(ADP-ribosyl)ated proteins were not identified in this study. The levels of protein poly(ADP-ribosyl)ation were correlated with inhibitory effects on cell growth, suggesting that the tankyrase 1 enzymatic activity is responsible for the growth defect caused by TNKS1 overexpression. This growth retardation phenotype can be used for detecting inhibitory activity against tankyrase 1, because inhibition should alleviate the growth phenotype.

3.2. Screening for compounds that rescue the TNKS1-induced growth arrest

We developed a liquid assay method for drug screening in a high-throughput manner, using the strain that overexpresses the N-type TNKS1 gene (see Section 2; Supplementary Fig. 1). We carried out high-throughput screening of ~7000 purified chemicals from the RIKEN NPDepo library, searching for compounds that rescue cell growth. We monitored cell growth using a WST-1 colorimetric assay. Flavone was identified as a compound that alleviates the yeast growth retardation (Supplementary Figs. 1 and 2A). The effective concentration of flavone was ~10 μM, much lower than that of the pan-PARP inhibitor 3AB (~10 mM) (Fig. 2B). We next examined whether flavone and 3AB decreased the PAR level of proteins, using yeast whole-cell lysates prepared from cells overexpressing the F-type TNKS1 gene; poly(ADP-ribosyl)ation was more evident in these cells than in cells overexpressing the

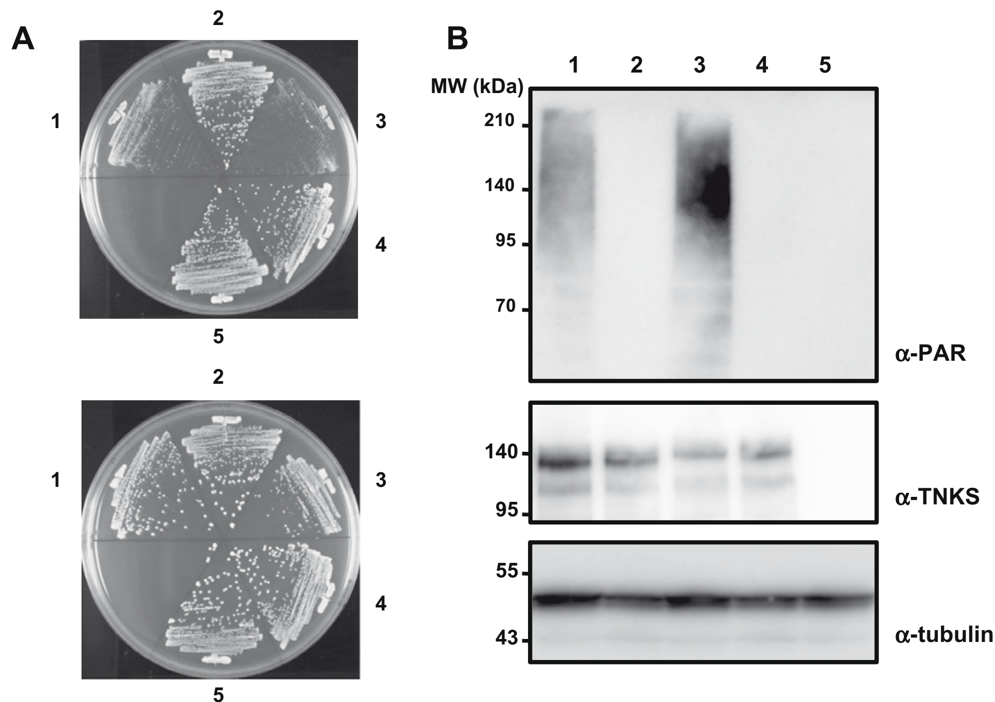

Fig. 1. Overexpression of the human tankyrase 1 gene caused growth defect and poly(ADP-ribosyl)ation in fission yeast. (A) Growth defect of the TNKS1 overexpression strains. The fission yeast cells harboring the N-type or F-type TNKS1 (WT or mutant H1184A) were streaked on MM (for overexpression; upper panel) and SD (for repression; lower panel) and incubated at 30 °C for 3 days. 1: N-type WT TNKS1; 2: N-type inactive mutant TNKS1; 3: F-type WT TNKS1; 4: F-type inactive mutant TNKS1; and 5: empty control. (B) Western blot analysis of yeast whole-cell lysates. Cells were grown in MM media for 22 h and harvested to prepare lysates. The lysates were subjected to Western blot analysis with anti-PAR, anti-tankyrase, and anti-tubulin antibodies.

Fig. 2. Effects of flavone and a pan-PARP inhibitor 3AB on the growth of TNKS1 overexpression cells and poly(ADP-ribosyl)ation of yeast proteins. (A and B) Cells overexpressing the N-type TNKS1 were treated with various concentrations of flavone (A) and 3AB (B). After an 18-h incubation of 50 μL of cell culture with various concentrations of drugs as indicated, we assessed cell growth using WST-1. (C and D) Effects of flavone and 3AB on poly(ADP-ribosyl)ation were analyzed by detecting PAR levels of proteins, using yeast cell lysates. Cells overexpressing the F-type TNKS1 were grown in MM media containing flavone (C) or 3AB (D) at various concentrations as indicated for 18 h, then harvested and analyzed with Western blotting.

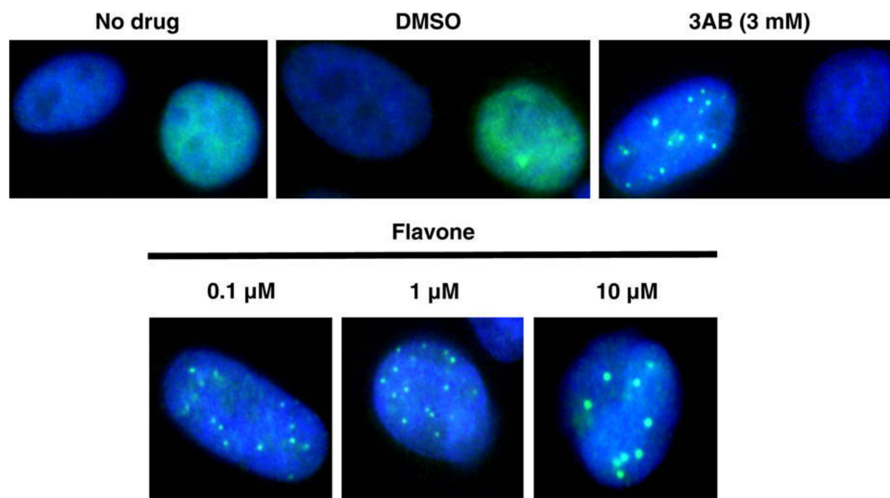

Fig. 3. Assembly of tankyrase 1 at the telomere regions by flavone in mammalian cells. Cells transfected with FN-tankyrase 1 construct were treated with flavone, 3AB, and DMSO at concentrations as indicated. Tankyrase 1 was detected using indirect immunofluorescence staining with the anti-FLAG antibody (shown in green). DNA was detected by staining with DAPI (shown in blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

N-type TNKS1 (see Fig. 1B). In agreement with effects on the cell growth, both flavone and 3AB almost completely inhibited poly-(ADP-ribosyl)ation of yeast proteins at concentrations of 5 μM and 10 mM, respectively (Fig. 2C and D). It has previously been reported that flavone inhibits PARP1 [23]. We also confirmed flavone's inhibitory activity against PARP1 by ELISA (Supplementary method and Supplementary Fig. 2). However, much higher concentrations of flavone were required to inhibit PARP1 activity.

3.3. Treatment with flavone causes punctate localization of tankyrase 1

To evaluate the tankyrase 1-inhibitory activity of flavone in mammalian cells, we examined the effect of flavone on the subcellular localization of tankyrase 1, using transient overexpression of TNKS1 in mammalian cells and indirect immunofluorescence staining [22]. This assay is based on the principle that TRF1 dissociates from telomeres in cells overexpressing tankyrase 1. When the TNKS1 gene was overexpressed in the nucleus, tankyrase 1 is flatly distributed in the nucleoplasm (Ref. [22] and Fig. 3). However, inhibition of tankyrase 1 by 3AB (3 mM) stabilizes the tankyrase 1–TRF1 complexes on telomeres, which can be visualized as punctate nuclear dots of exogenous tankyrase 1. Upon treatment with flavone, significant punctate staining of the tankyrase 1 foci appeared under concentrations ranging from 0.1 to 10 μM. These results indicate that flavone inhibits tankyrase 1 not only in yeast but also in mammalian cells.

4. Discussion

The PARP family of enzymes has attracted attention as targets of therapeutic drugs, and several assay systems have been established. Indeed, screening for small-molecule inhibitors against two PARP family members, PARP1 and PARP2, was previously reported in budding yeast [24]. Since tankyrase 1 is a therapeutic target for cancer therapy, especially for BRCA-associated cancers, it was important to develop of a facile screening method applicable to high-throughput assay. In this study, we established a phenotypic screening system for tankyrase 1 inhibitors, using genetically engineered fission yeast strains, and demonstrated its usefulness by screening a chemical library and identifying flavone as a tankyrase 1 inhibitor. Although the tested compounds supplied from RIKEN NPDepo library contain several flavonoids, only flavone was identified as a hit compound in this screening, suggesting that flavone is more potent in inhibiting tankyrase 1 than other flavonoids in the library. Flavone was reported as an inhibitor of PARP1 by Geraets et al. [23]. In their *in vitro* assay, the activity of PARP1 was inhibited by 100 μM flavone; the inhibition was slightly stronger than the inhibition by 3AB at the same concentration [23]. These findings are consistent with our ELISA (Supplementary Fig. 2). Because the effective concentration of flavone for inhibiting tankyrase 1 activity (0.1–10 μM) (see Fig. 3) was much lower than for PARP1, flavone may preferentially inhibit tankyrase 1 rather than PARP1 *in vivo*.

When human tankyrase 1 was overexpressed in fission yeast having no intrinsic PARPs, poly(ADP-ribosyl)ation of yeast proteins was observed, although these proteins have yet to be unidentified. The conventional method for measuring tankyrase activity is still based on a radioactive format, whereas a non-radioactive ELISA method has been established for PARP1. It should be noted that in our method, drug effects on PAR activity of tankyrase are easily detectable by Western blot analysis using yeast cell lysates, and can be detected without use of a radioisotope-labeled substrate (NAD^+). Thus, a yeast cell-based drug screening using gene overexpression can overcome potential difficulties in establishing a high-throughput assay for tankyrases, and has great potential for identifying and developing inhibitors of tankyrases and other PARP family enzymes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.03.021.

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