Novel Inhibitors of Poly(ADP-ribose) Polymerase/PARP1 and PARP2 Identified Using a Cell-based Screen in Yeast

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ABSTRACT

Multicellular organisms must have means of preserving their genomic integrity or face catastrophic consequences such as uncontrolled cell proliferation or massive cell death. One response is a modification of nuclear proteins by the addition and removal of polymers of ADP-ribose that modulate the properties of DNA-binding proteins involved in DNA repair and metabolism. These ADP-ribose units are added by poly(ADP-ribose) polymerase (PARP) and removed by poly(ADP-ribose) glycohydrolase. Although budding yeast Saccharomyces cerevisiae does not possess proteins with significant sequence similarity to the human PARP family of proteins, we identified novel small molecule inhibitors against two family members, PARP1 and PARP2, using a cell-based assay in yeast. The assay was based on the reversal of growth inhibition caused by the heterologous expression of either PARP1 or PARP2. Validation of the assay was achieved by showing that the growth inhibition was relieved by a mutation in a single residue in the catalytic site of PARP1 or PARP2 or exposure of yeast to a known PARP1 inhibitor, 6(5H)-phenanthridinone. In separate experiments, when a putative protein regulator of PARP activity, human poly(ADP-ribose) glycohydrolase, was coexpressed with PARP1 or PARP2, yeast growth was restored. Finally, the inhibitors identified by screening the yeast assay are active in a mammalian PARP biochemical assay and inhibit PARP1 and PARP2 activity in yeast cell extracts. Thus, our data reflect the strength of using yeast to identify small molecule inhibitors of therapeutically relevant gene families, including those that are not found in yeast, such as PARP. The resultant inhibitors have two critical uses (a) as leads for drug development and (b) as tools to dissect cellular function.

INTRODUCTION

Living organisms possess mechanisms to regulate cell cycle progression and to preserve genomic integrity. Failure of these mechanisms in multicellular organisms results in disorders ranging from the unregulated cell proliferation associated with cancer to massive cell death after the fall of tissue oxygen and glucose levels in cardiac or brain ischemia (1).

A key cellular response to genomic damage is the posttranslational modification of nuclear proteins in response to DNA strand breaks (2, 3). One known modification is the addition to specific proteins of up to 200 residues of ADP-ribose to form branched polymers. These polymers act as binding sites for repair proteins that play a central role in DNA metabolism (4). The enzyme responsible for the addition of these polymers is PARP. PARP1 associates with DNA and with chromatin-binding proteins such as histones, transcription factors, and key DNA repair proteins (4). Although a number of nuclear proteins such as histones are substrates for PARP1, a major substrate is PARP1 itself, as demonstrated by the BRCA1 COOH-terminal homology region (4). Regulation of automodification of PARP1 is twofold: through PARP1-DNA interactions and PARP1-PARP1 dimerization (5). PARP1 acts together with the DNA damage repair system to regulate DNA base excision repair, apoptosis, and necrosis (4). Studies of mouse strains lacking the PARP1 gene have identified two roles for this encoded protein, depending on the extent of DNA damage (6–8). Moderate damage elicits a protection response similar to that observed for checkpoint genes, leaving PARP1 knockout mice vulnerable to γ-irradiation and alkylating reagents (8). In cases of extensive DNA damage, PARP1 activity depletes cellular energy pools, which eventually leads to cell death (3, 6).

PARP1 also has a putative role in signaling DNA damage and in recruiting proteins to sites of double-strand breaks. This hypothesis was based on the ability of proteins, such as p53 and other repair enzymes, to bind to the poly(ADP) polymers present on PARP1 (4, 9). PARP1 inhibitors exaggerate the cytotoxic effects of DNA damage by limiting the ability of cells to regulate DNA base excision repair. In this role, PARP inhibitors are being tested as chemosensitizing agents during cancer chemotherapy (10, 11).

Another response to more extensive DNA damage mediated by PARP1 is the promotion of cell death, as seen in cases of ischemic injury (12). This process can occur when PARP1 activation is highly stimulated and thus consumes large amounts of NAD, the source of ADP-ribose. This condition depletes the cellular energy stores (13). PARP1 knockout mice are highly resistant to ischemia during steptozocin-induced type I diabetes, myocardial infarction, stroke, and neurodegeneration (6, 14). In support of a role for PARP1 in cell death in various inflammation processes, several studies have shown protection against cellular injury in numerous target cells by using known PARP1 inhibitors (12).

For many years PARP1 has been the only known PARP. However, modification of cellular proteins with ADP-ribose polymers still occurs in PARP1 knockout mice, suggesting the presence of other proteins with PARP activity (15). Indeed, new members of the PARP family have been identified based on the presence of domains that share considerable sequence similarity with the catalytic domain of PARP1 (16–20). Although some members of the PARP family do not possess a well-defined Zn<sup>2+</sup> finger DNA-binding motif or an auto-modification domain such as that described for PARP1, they still catalyze the formation of ADP-ribose polymers in a DNA-dependent manner and are capable of automodification (16, 17).

Two additional members of the PARP family are tankyrase and VPARP (18, 20). Tankyrase is associated with the telomerase complex that acts to regulate telomere length at replication, and VPARP is a component of a multisubunit complex referred to as a “vault” (21–23). The name vault is based on its observed structure by electron microscopy (22). The cellular location of VPARP is predominantly cytoplasmic; however, there is a small fraction associated with the mitotic spindle (18). Unlike PARP1, tankyrase and VPARP are not activated by DNA damage (18, 20). Tankyrase modifies the telomere-binding protein TRF1 in vitro (15). TRF1 stabilizes the ends of chromosomes, and it has been proposed that modification of TRF1 with ADP-ribose polymers serves to regulate its ability to form a loop.
structure at chromosome ends (15, 23). In other studies, tankyrase has been shown to promote telomere elongation in human cells (24). A substrate of VPARP is the major vault protein, MVP (it is also capable of automodification); these complexes are up-regulated in multidrug-resistant cancer cell lines (25, 26). The various cellular locations and domain structures of the PARP family members strongly suggest that they have distinct cellular roles. Identification of selective inhibitors might help elucidate the function of these enzymes.

Poly(ADP-ribose) polymers can be removed by PARG, a member of a large family of related enzymes (27–30). This enzyme is thought to regulate the cellular function of PARP family members by removing ADP-ribose units, which results in changes in the branching pattern of the polymers (15, 27). There is some evidence to support the hypothesis that polymer syntheses by different PARP orthologues might be hydrolyzed by specific PARGs (27). Although a complete understanding of the physiological activities of PARPs remains unclear, inhibitors of the activity of PARP1 and related proteins could provide new therapeutic approaches to both cancer and ischemia caused by reperfusion injury and inflammatory processes (12).

Kaiser et al. (31) found that the constitutive expression of PARP1 in Saccharomyces cerevisiae is only possible with simultaneous inhibition of ADP-ribosylation activity through the addition of the known inhibitor 3-methoxybenzamide to the growth media. Induction of fully active PARP1 under the conditional galactose promoter led to growth arrest (31, 32). The growth inhibition was relieved by removal of the NH2-terminal region of PARP1 that contains the DNA-binding domain. Yeast does not possess endogenous PARP1 activity (32), so the cause of the growth arrest is unknown. Antibodies raised against polymers of poly(ADP-ribose) were used to determine that a protein of approximately 116,000 kDa, the size of PARP1 itself, was ADP-ribosylated in yeast cells expressing PARP1 (31). This led to the proposal that growth inhibition in yeast might occur through sequestration of chromosomal proteins by the ADP-ribose-decorated PARP1 protein rather than toxicity due to direct modification of a yeast protein by PARP (31).

In this work, we demonstrate that PARP2, a protein closely related to PARP1, also causes growth inhibition when conditionally expressed in yeast. The reduced growth phenotype was used as an assay to screen for novel inhibitors of these proteins by selecting those compounds that can restore growth to yeast expressing PARP1 or PARP2. The inhibitors identified in our screen also inhibit recombiant PARP1 activity in vitro. Certain inhibitors show selectivity for PARP1 or PARP2. These compounds are the first to be reported that specifically recognize PARP orthologues in cells. Our data demonstrate the utility of yeast as a screening system to identify inhibitors of human genes and gene families, even when the protein is not highly conserved with any yeast proteins. Thus, our purpose is not to understand the interaction of heterologous genes with endogenous yeast proteins. Rather, we use the “biochemical activity” of the proteins expressed in yeast to obtain inhibitors that can then be used as tools to dissect function in a relevant mammalian assay.

MATERIALS AND METHODS

Yeast Strains, Media, and Methods. Isogenic derivatives of the W303 S. cerevisiae background were used in this study (33, 34) and are listed below. Yeast strains lacking the major efflux pumps PDR5 and SNQ2 were constructed from strain YM4 (a gift of T. Lilja, Microcide Pharmaceuticals) deleted for both via a two-step gene disruption process (35). The pdr5Δ allele was deleted at bp 101 through 4149 of the PDR5 open reading frame, and the snq2Δ allele was deleted for bp 235 through 4105 of SNQ2, resulting in strain YPB63. To construct strain EIS20-2B, YPB63 was backcrossed to W303-1a, and retention of the pdr5Δ and snq2Δ alleles was confirmed by whole cell PCR and increased sensitivity to cycloheximide, a toxic pump substrate (data not shown). YAN100 is an EIS20-2B derivative that contains a complete deletion of the HIS3 gene. The yeast media protocols and genetic molecular biology techniques used for these studies are standard protocols (36).

The genotype of the strains is as follows: (a) EIS20-2B and YPB63; MatA ade2-1 his3-1115 leu2-3,112 trpl-1, ura3-1, can1-100, pdr5Δ, snq2Δ; (b) YAN100, MatA ade2-1 his3Δ; KanMX, leu2-3,112 trpl-1, ura3-1, can1-100, pdr5Δ, snq2Δ; (c) W303α(w3-301-1B); MatA ade2-1 his3-1115 leu2-3,112 trpl-1, ura3-1, can1-100, pdr5Δ, snq2Δ; and (d) YM4, MatA ade2-101 his3Δ200 leu2-3,112 trpl-1 ura3-52.

Cloning and Analysis of PARP1, PARP2, and PARG. The cDNAs corresponding to the complete open reading frames of PARP1 and PARP2 were PCR-amplified from pooled total cDNAs initially synthesized from placental, fetal brain, and fetal liver polyadenylated mRNAs (Clontech). First-strand cDNA synthesis and second cDNA synthesis PCR was performed using the in vitro reverse transcriptase (Life Technologies, Inc., Solana Beach, CA) high-fidelity thermostable DNA polymerases according to the manufacturers’ instructions. All oligonucleotides were designed to amplify their target cDNAs and carry approximately 40 bp of homology at their 5’ end with the yeast expression vector pYES2 (33). The pYES2 vector (Invitrogen, Carlsbad, CA) contains the yeast GAL1 promoter, CYC1 transcription terminator, 2 μ replication origin, and the URA3 gene.

After PCR, the amplified target cDNAs were agarose gel-purified. Approximately 30 ng of target cDNA were co-transfected with approximately 200 ng of EcoRI-digested pYES2 into W303α, and transformants were selected for uracil prototrophy. Homologous recombination of the target cDNAs into pYES2 was confirmed by yeast whole cell PCR. After the synthetic lethal phenotype of the expressed PARP1 and PARP2 was verified, plasmids from at least three independent transformants were subsequently rescued into the Escherichia coli strain XL1-10-GOLD (Strategene) by electroporation and further characterized by restriction enzyme analysis and DNA sequencing of the 5’ and 3’ cloning junctions. These studies verified the presence of the desired structures.

During the course of this work, it was noted that the pYES2-PARP2 plasmid in E. coli was toxic (data not shown), thus the primer SYSDP2lac incorporated a lacO repressor-binding site: this established the plasmid during its propagation in XL1-10-GOLD (which carries the lacI gene). This vector construction still exhibited instability during growth in yeast. Thus the GALI-lac-PARP2-CYC1 terminator cassette was recombinationally cloned into yeast URA3 2 μ vector Yep24 (38) using the oligonucleotides Y24smacyct (5’-TACAAATTAGGCTTCAAATTTATATCATGTTATTACCCGCCG-GGCGGGCCCGAAATATACCGCTTGACG) and Y24pvu2gal (5’-GGGC-GAGCCGCCGAGGAGATTCGAACTAGGAGTTGCGACTCCGAGC), respectively. The oligonucleotide SYSDP2lac contains the lacO operator site. PCR amplification was carried out using either Bio-X-ACT (Bioline USA Inc., Kenilworth, NJ) or pfTurbo (Strategene, La Jolla, CA) high-fidelity thermostable DNA polymerases according to the manufacturers’ instructions. All oligonucleotides were designed to amplify their target cDNAs and carry approximately 40 bp of homology at their 5’ end with the yeast expression vector pYES2. The pYES2 vector (Invitrogen, Carlsbad, CA) contains the yeast GALI promoter, CYC1 transcription terminator, 2 μ replication origin, and the URA3 gene.

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For compound screening and subsequent genetic testing, the PARP1 and PARP2 expression cassettes were integrated into the indicated strains using the pARC series of dual episomal/integrative plasmid constructs. A brief summary will be presented in lieu of a detailed description of the steps used to construct the pARC series of vectors. The vectors are derived from the pRS series of vectors (39, 40). The basic elements of the pARC plasmids include a 713-bp SplbHami fragment containing the S. cerevisiae GALI promoter and a 243-bp BglII/HindIII fragment containing the GAL4 terminator region. This region surrounds a 45-bp polylinker that contains unique sites for SalI, SphI, XhoI, and Aval and replaces the pRS polylinker; the base vector and polylinker of the pRS vectors is pBlueScript II. Both CEN- and 2μ-based vectors were generated with 44 bp of the 5’ end of the LYS2 gene (bp 8-52 of 4176

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the LYS2 ORF) and 43 bp of the 3' end of the LYS2 gene (bp 4133–4176 of the LYS2 ORF) flanking the CEN-ARSH4 element or the 2μ replication origin. In addition, 5fl sites flank the CEN-ARSH4 or 2μ elements, such that digestion with this restriction enzyme liberates the elements and allows the subsequent integration into the endogenous LYS2 locus via a y deletion mechanism (40). For this work, integrants were selected for α-aminoadipate resistance and by marker prototrophy, e.g., Leu+ or Ura+. Integration was confirmed by PCR.

For integration of PARP1, a SpeI/MluI (the MluI site made blunt with Klenow) GAL1-PARPI-CYC1 terminator fragment from pYES2-PARPI was ligated into a SpeI/EcoRI-cut pARC35A, a CEN-ARSH4 LEU2 vector containing the original pBluescript II polylinker; these steps resulted in plasmid pARC35APARPI. Digestion with SfiI allowed integration of the PARP1 expression cassette into the LYS2 locus. For integration of PARP2, the GALI-PARP2-CYC1 terminator fragment from pYES2-PARPI was amplified by PCR using puTurbo with primers YSPRC (5'-GATGTTAAATGAGAAGAATTGAGATGTCAGTCACAGCAGTTGTAATAGCGTG-3') and YSPCC (5'-TAAATGGATCCCTATTTAGCTAAGGGAGAAGAATACTTAATCCGCCCCTGGA-3'), a primer containing homology at the 5' end to the GAL4 terminator) and GLUAS1 (5'-TGAATCAGCAGATTAAAGCGCCGCGG, a primer with homology to the GAL1 UAS element). This fragment was cotransformed into yeast with XhoI-digested pARC25B (2μ LEU2 GAL1 promoter and GAL4 terminator). As before, the recombimants were verified by phenotypic analysis and PCR before rescue into E. coli. The resulting pARC25BPAPPl plasmid was digested with SfiI and integrated into the LYS2 locus.

Phenotypic expression of the PARP1 and PARP2 clones was confirmed by constructing green fluorescent protein fusions to the COOH-terminal end of the proteins using green fluorescent protein-KanMX cassettes (41–43). Expression was assessed via fluorescence microscopy and Western blot analysis. All active site mutations were confirmed by sequencing.

PARG was isolated and cloned using primers 5YSPAR (5'-GGATTAT-TACCCCTATACCTTTAAAGCGTGCAAAACACGCTATGCGGCGCGC-3') and 3YSPAR (5'-GGATTATACCGTGGAACTACTAATCCTATTTAGCTAAGGGAGAAGAATACTTAATCCGCCCCTGGA-3') to amplify the complete ORF from a testes cDNA library (37). The purified PCR product was cotransformed with digested pYES2 plasmid into EIS20-2B, and the resulting recombinants were confirmed using whole cell PCR. Transformed isolates were subsequently reisolated and transformed into E. coli XL10-GOLD and confirmed by restriction enzyme analysis resulting in plasmid pYES2-PARG. To assess whether expressed PARG could suppress the synthetic lethality caused by PARPI and PARP2, plasmid pYES2-PARG was transformed into EIS20-2B cells containing an integrated chromosomal copy of PARP1 or PARP2. Purified transformants were subsequently inoculated into galactose-containing media, and their growth was assayed.

PARP Activity Assays. A PARP Activity Assay Kit (Treven, Inc., Gaithersburg, MD) was used to measure the functional activity of human PARP1 and PARP2. Human PARP1 (supplied in the PARP Activity Assay Kit from Treven, Inc.) or crude cell extracts of yeast expressing a cDNA encoding the full-length human PARP1 or PARP2 gene were used in the assay. Activity was measured by determining the level of incorporation of radiolabeled NAD in a TCA-precipitable polymer composed of ADP-ribose units.

Activity was measured by determining the level of incorporation of radiolabeled NAD in a TCA-precipitable polymer composed of ADP-ribose units. The timed enzymatic reaction was initiated by the addition of the cell lysates, incubated at room temperature, and stopped by the addition of 20% TCA to precipitate ribosylated proteins. The protein precipitate was suspended in liquid scintillation fluid and analyzed using a scintillation counter. Each reaction was done in triplicate, and the results are shown as the mean ± SEM.
Lacking uracil. As a control, yeast carrying an integrated copy of PARP1 or PARP2 expressed in yeast, we conditionally coexpressed PARG to determine whether PARG reverses the growth defect caused by human PARP1 or PARP2. Fluorescence microscopy revealed that expressed mammalian PARP1 and PARP2 accumulated in the nucleus (data not shown).

Yeast cell growth was severely inhibited when PARP1 and PARP2 were expressed episomally or from a chromosomal locus (Figs. 1 and 2). In our initial characterization of the growth phenotype caused by PARP1 or PARP2, the genes were episomally expressed but were integrated for compound screening.

Expression of Human PARG in Yeast Reverses the Growth Inhibition Caused by PARP1 or PARP2 Expression. To determine whether PARG reverses the growth defect caused by human PARP1 or PARP2 expressed in yeast, we conditionally coexpressed PARG from a high-copy plasmid in yeast cells carrying an integrated copy of PARP1 or PARP2 also under control of the inducible galactose promoter. Yeast cells were grown in glucose medium (uninduced) and then switched to 2% galactose (induced) for 40 h in synthetic media containing 2% galactose to induce PARG expression. PARG expression was analyzed in the presence of episomally expressed PARG also under control of the GAL1 promoter or a vector control.

Expression of PARG in yeast alleviates the growth inhibition caused by PARP1 or PARP2 expression. Yeast cells carrying integrated copies of PARP1 or PARP2 under control of the inducible GAL1 promoter were grown in glucose or 2% galactose. Their growth was analyzed in the presence of episomally expressed PARG also under control of the GAL1 promoter.

Sensitivity of Yeast to Analogues of 6(5H)-phenanthridinone Is Increased in Strains Lacking the Two Major Efflux Pumps, PDR5 and SNQ2. To optimize the sensitivity of the yeast strain to the screening compounds, mutations were made in the two efflux pumps, PDR5 and SNQ2 (45). Pdr5p and Snq2p are the major efflux pumps in S. cerevisiae that confer resistance to several unrelated fungal growth inhibitors. To examine whether yeast strains lacking these two major transporters were more sensitive to the active analogues of 6(5H)-phenanthridinone, the activity of the analogues with respect to their ability to restore growth to yeast expressing PARP1 was evaluated in pdr5Δ and snq2Δ strains (YPB63, which lacks the two pumps) or (W303, wild-type for PDR5 and SNQ2; see Fig. 5).

Whereas four analogues showed a similar effect on growth restoration regardless of the presence or absence of the two efflux pumps (data not shown), yeast lacking the efflux pumps were more responsive to 6(5H)-phenanthridinone, ICX56216703, ICX56259835, and ICX56274004. Not surprisingly, as in the case of the known PARP1 inhibitor compound 6(5H)-phenanthridinone, the three active analogues also restored the growth of PARP2-expressing yeast to a similar degree.

Potential Selectivity Is Observed among the Compounds Identified as Hits in the Yeast Cell-based Screen. To identify new classes of inhibitors, we screened the yeast strain carrying integrated...
6(5H)-Phenanthridinone, computationally chosen analogues, and most of the screening hits show similar efficacy on PARP1 and PARP2. The growth inhibition caused by PARP1 expression in yeast was used to screen for novel inhibitors of this protein by selecting those compounds that can restore growth to yeast expressing PARP1. Ten hits were identified, and their structures are displayed in the figure, along with that of the known inhibitor 6(5H)-phenanthridinone and a few computationally chosen analogues. The structure of the compounds tested is shown along with the EC_{50} value for both PARP1- and PARP2-expressing cells. ND (not determined) indicates that the compound was not tested against PARP2-expressing cells. NA (not active) indicates that the compound has no effect on growth restoration of yeast up to the highest concentrations tested (128 μM). For some compounds, an accurate EC_{50} could not be determined because at the highest compound concentrations tested (limited by solubility), the growth restoration was linear, making a curve fit to a four-parameter logistic equation impossible.

Yeast lacking the two major efflux pumps PDR5 and SNQ2 show increased sensitivity to screening compounds. Normal yeast strains or strains carrying a deletion of the two efflux pumps PDR5 and SNQ2 and expressing PARP1 were grown in galactose medium to induce PARP1 expression. The strains were exposed to increasing concentrations of the known inhibitor 6(5H)-phenanthridinone and two analogues, ICX56242099 and ICX56209576. The level of growth restoration at different concentrations of the compounds is plotted.
PARP1 against a 16,000-member pilot library of small organic “drug-like” compounds for their ability to restore growth while PARP1 was expressed (in the presence of galactose). Ten hits showed dose-dependent growth restoration on cells expressing PARP1 (see Figs. 4 and 7; the computationally chosen analogue ICX56259835 was also identified as a screening hit). We then tested the ability of the confirmed hits to restore growth to yeast expressing PARP2. Those values in Fig. 4 labeled NA were compounds that were found to have no activity against PARP2 at the highest concentration tested (128 μM), and ND (not determined) refers to those compounds that were not tested under the specific conditions in Fig. 4. Unlike the results observed for the analogues of 6(5H)-phenanthridinone, some of the 10 compounds identified by screening showed patterns suggestive of selectivity for PARP1 or PARP2 (Fig. 4).

One inhibitor class showed modest potency (ICX56225770, ICX56244215, and ICX56280834), whereas one compound in this group, ICX56290675, had a higher potency and showed some selectivity for PARP1. ICX56222404 and ICX56259537 are novel inhibitors with more pronounced selectivity for PARP1. Finally, ICX56298231 showed potential selectivity for PARP2 (Fig. 6). Although a difference in the level of growth restoration was observed for PARP1 versus PARP2 in the presence of ICX56258231, an accurate EC_{50} value for PARP1 could not be determined. Normally this value is derived from a curve fit to a four-parameter logistic equation, but in this case, at the highest concentration of soluble compound, a plateau in the growth restoration curve was not reached.

Compounds That Restored Growth in the Yeast Cell-based Assay Inhibit Purified PARP1 in Vitro. To clarify whether the small molecules identified in screening directly inhibited PARP1 activity, the hits were tested for their ability to inhibit human recombinant PARP1 in a biochemical assay. This assay measures the incorporation of radiolabeled ribose derived from NAD into PARP1. Baseline activity was established by measuring incorporation from the radiolabeled ribose in the absence of the inhibitors.

Dose-response curves were constructed for three of the compound hits (ICX56304405, ICX56290675, and ICX56258231) and included 6(5H)-phenanthridinone and its inactive analogue, ICX56225328 (Fig. 7). These results show clearly that the compounds identified in the yeast screen are direct inhibitors of PARP1.

Compounds That Restored Growth Activity Also Inhibit the Activity of PARP1 and PARP2 from Yeast Cell Extracts. The activity of PARP1 or PARP2 in extracts from cells grown to log phase over 8 h in 2% glucose (uninduced) was negligible. However, cells grown under similar conditions but in the presence of 2% galactose (induced) showed an increase of 20-fold for PARP1 activity and 10-fold for PARP2 activity (Fig. 8). The screening compounds were then tested for their ability to inhibit the activity of PARP1 and PARP2 observed in the yeast extracts. All the compounds inhibited PARP activity as effectively as the known inhibitor with the exception of the inactive analogue, ICX56225328 (Fig. 9).

DISCUSSION

PARP1 and PARP2 cause an interference in cell growth when expressed in yeast that can be alleviated by making a single point mutation in a conserved residue within the active site or by exposing the cells to the known inhibitor 6(5H)-phenanthridinone. The glycohydrolase PARG, which removes ADP-ribose units from PARP in vivo, also reverses the growth phenotype when coexpressed with PARP1 or PARP2 in yeast. We have identified a number of small molecule inhibitors of the mammalian proteins PARP1 and PARP2 using a yeast cell-based assay based on growth interference. Our data show that the growth defect observed in yeast as a function of PARP1 or PARP2 expression is dependent on their catalytic activity, thereby constituting valid screens for small molecule inhibitors of PARP and its family members. The small molecules that have been identified by their ability to alleviate the growth interference caused by PARP expression in yeast also inhibit mammalian PARP in a biochemical assay and PARP1 and PARP2 activity in yeast cell extracts.

The chemical inhibitors found in this study confirm previous reports of small molecule PARP1 inhibitors found using other methods and demonstrate the possibility of previously unreported selective inhibition of PARP isoforms. The phenanthridinone analogues selected from our corporate library using a chemoinformatic approach were mainly of the phthalazinone class (Fig. 4). This nonselective class of inhibitors has been described previously and served to confirm and validate our screening approach. More interesting compounds were uncovered in the pilot screen (Fig. 6) in terms of both
PARP1 requires DNA binding in yeast. Perhaps chromosomal breaks interfere in yeast. These results imply that the catalytic activity of nuclear localization of PARP1, but can no longer cause growth containing the DNA-binding domain of PARP has no effect on the catalytic activity of PARP1 in mammalian cells is activated by double-strand DNA breaks. What activates PARP1 and PARP2 in yeast, that occur during normal metabolism, replication, and mitosis stimulate PARP activation in yeast. In mammalian cells, when DNA damage is severe, high levels of ADP-ribosylation result in a depletion of cellular NAD stores. Low NAD levels result in a failure of cellular metabolism and cell death through apoptosis (3, 4). However, previous investigators have shown that the NAD levels of yeast remain unchanged when PARP is expressed, suggesting that the growth interference is not due to depletion of NAD levels and subsequent effects on cellular metabolism (31, 32).

Many researchers have expressed foreign proteins in yeast to identify those that functionally complement a yeast mutation (46–48). This approach requires the metabolic pathway affected by the yeast mutation to overlap with a conserved pathway in metazoans. The ability of a foreign protein to replace a yeast protein allows one to study variants of the human protein in a genetically tractable organism. These variants can be in the form of mutants up-regulated in a specific disease tissue or genes containing nucleotide polymorphisms.

However, yeast is not limited to the study of processes that are conserved between metazoans and yeast. Investigators have shown that yeast can be used to identify novel members of the apoptotic pathway, even though no such process exists in unicellular organisms (49–52). These investigators were able to identify two mammalian genes, using a screen based on growth interference in yeast, that inhibit the induction of apoptosis in mammalian cells (49, 52). As in the case for BAX and BCL2, the yeast genome does not encode a protein with similarity to PARP.

Growth interference phenotypes have not been as widely used as complementation assays for compound screening. A benefit of our screen is the elimination of false positives caused by toxic compounds because toxic compounds do not restore growth in yeast. One criticism is that growth interference phenotypes caused by the expression of a mammalian cDNA might not reflect the normal activity of the encoded protein. Therefore, screening such an assay for compounds that reverse the growth interference phenotype might not be relevant if they do not inhibit the mammalian protein but inhibit a yeast protein instead. We have routinely shown that potential selectivity of inhibition and chemical structure. The known phthalazine (ICX56225770) and quinazoline (ICX56244215, ICX56280834, ICX56290675, and ICX56304405) inhibitor classes showed modest potency. For one compound, ICX56290675, some selectivity for PARP1 was observed. The thiochromone ICX56222404 and the benzothiazinone ICX56259537 are novel inhibitors with what appears to be more pronounced selectivity for PARP1. Finally, the results for the substituted phthalazine ICX56258231 suggest selectivity for PARP2. As discussed above, we have determined that the growth interference caused by PARP1 and PARP2 is due to their poly(ADP)-ribosylation activity. Normally the catalytic activity of PARP1 in mammalian cells is activated by double-strand DNA breaks. What activates PARP1 and PARP2 in yeast and the mechanism of the observed growth inhibition are less clear. Kaiser et al. (31) have shown that truncation of the NH2 terminus containing the DNA-binding domain of PARP has no effect on the nuclear localization of PARP1, but can no longer cause growth interference in yeast. These results imply that the catalytic activity of PARP1 requires DNA binding in yeast. Perhaps chromosomal breaks
active site mutations eliminate the growth interference in yeast caused by a wide variety of mammalian enzyme types; the active site mutation thus directly connects the biochemical activity of the introduced mammalian protein to its growth-interfering activity. In addition, we have tested a number of different families of mammalian proteins and found many that cause growth interference in yeast, e.g., kinases, small GTPases, and guanine exchange factors (data not shown). For those proteins for which a biochemical assay is available, we have tested the screening hits and observed that they act directly on the human protein;6 offentliches the rank order potency is preserved.

Our study demonstrates the usefulness of yeast growth interference assays for the identification of chemicals that block the activity of a human protein causing the growth defect. This same approach was used to screen for inhibitors of the influenza M2 ion channel, a viral protein that results in lethality when expressed in yeast (53). We have used cell-based assays to identify compounds that inhibit two members of the PARP family. We have shown that using these assays, we can identify compounds that specifically inhibit the biochemical activity of the mammalian protein in vitro and from yeast cell extracts. By using two members of the PARP family, we have also shown that our assays are sensitive enough to detect structural activity differences between a few compounds. Thus, the yeast cell-based assay is excellent as a means to assay mammalian proteins, even if there is no counterpart in yeast. These assays potentially allow differentiation of compound sensitivity of family members and thus define structure-activity relationships important in the development of specific inhibitors.

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Unpublished observations.


Novel Inhibitors of Poly(ADP-ribose) Polymerase/PARP1 and PARP2 Identified Using a Cell-based Screen in Yeast

Ed Perkins, Dongxu Sun, Allen Nguyen, et al.


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