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Chemical genetic profiling of the microtubule-targeting agent peloruside A in budding yeast *Saccharomyces cerevisiae*

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ABSTRACT

Peloruside A, a microtubule-stabilising agent from a New Zealand marine sponge, inhibits mammalian cell division by a similar mechanism to that of the anticancer drug paclitaxel. Wild type budding yeast *Saccharomyces cerevisiae* (haploid strain BY4741) showed growth sensitivity to peloruside A with an IC_{50} of 35 μM. Sensitivity was increased in a *mad2*Δ (Mitotic Arrest Deficient 2) deletion mutant (IC_{50} = 19 μM). Mad2 is a component of the spindle-assembly checkpoint complex that delays the onset of anaphase in cells with defects in mitotic spindle assembly. Haploid *mad2*Δ cells were much less sensitive to paclitaxel than to peloruside A, possibly because the peloruside binding site on yeast tubulin is more similar to mammalian tubulin than the taxoid site where paclitaxel binds. In order to obtain information on the primary and secondary targets of peloruside A in yeast, a microarray analysis of yeast heterozygous and homozygous deletion mutant sets was carried out. Haploinsufficiency profiling (HIP) failed to provide hits that could be validated, but homozygous profiling (HOP) generated twelve validated genes that interact with peloruside A in cells. Five of these were particularly significant: *RTS1*, *SAC1*, *MAD1*, *MAD2*, and *LSM1*. In addition to its known target tubulin, based on these microarray 'hits', peloruside A was seen to interact genetically with other cell proteins involved in the cell cycle, mitosis, RNA splicing, and membrane trafficking.

1. Introduction

Determining the mode of action of new compounds is an important goal in drug development. Chemical genetics profiling in yeast provides a data set that allows chemical compounds and yeast genes to be organised into functionally relevant interacting groups or networks (Parsons et al., 2004, 2006). The availability of comprehensive deletion strain collections and gene libraries make yeast particularly well suited to large-scale genetic screens. Yeast deletion sets are available in which all non-essential genes have been knocked out by homologous recombination and insertion of an antibiotic resistance cassette. The cassette is flanked by two 20mer DNA sequence tags or "barcodes" (denoted as Up (UP) and Down (DN) tags), each unique to the deletion strain, allowing identification of the individual strains from a pool by PCR with common primers and hybridisation to a microarray of all tags (Giaever et al., 2002; Pierce et al., 2007; Winzeler et al., 1999). Microarray analysis can be used to determine which genes interact with a chemical of unknown function. One such microarray screen involves haploinsufficiency profiling (HIP) in which a heterozygous deletion set is used that has only one intact copy of each gene, with the other copy deleted. In the presence of a chemical that specifically inhibits the product of the heterozygous gene locus, growth is inhibited. A reduced copy number of a drug's target gene from two to one sensitises a diploid cell to the drug. The HIP microarray screen thus identifies the target of the drug (Giaever et al., 1999; Lum et al., 2004). HIP results provide comprehensive understanding of the genome-wide cellular response to chemical compounds with unknown mechanisms of action (Giaever et al., 2004; Lum et al., 2004). Homozygous deletion profiling (HOP) involves use of yeast haploid deletion sets in which no copy of a gene is present at a given locus or both copies of a diploid strain are deleted. Since no functional gene is present, only non-essential genes can be tested for interactions with the drug of interest. Following exposure to a drug, microarray HOP screens reveal functionally related or connected genes ('friends of the target') that buffer the cell against the cytotoxic or cytostatic effects of the drug.

Yeast have an effective set of drug efflux pumps that provide resistance to the deleterious effects of drugs. The efflux systems establish a pleiotropic drug resistance (PDR) phenotype similar to the multidrug resistance (MDR) phenotype of mammalian cells (Jungwirt

Abbreviations: HIP, haploinsufficiency profiling; HOP, homozygous deletion profiling; MDR, multidrug resistance; PDR, pleiotropic drug resistance; PelA, peloruside A

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and Kuchler, 2006). Typically, drugs that are effective in mammalian cells at nanomolar concentrations often are 100- to 1000-fold less potent in yeast, and this difference may be partly due to PDR mechanisms as well as differences in target affinity. Some yeast deletion sets are available that have the main PDR genes (*PDR1*, *PDR3*, *PDR5*) deleted, and these pump-deficient mutants often show increased sensitivity to test drugs.

Peloruside A (PelA) was first described by West et al. (2000) after isolation and purification from a New Zealand marine sponge, and its mode of action as a microtubule-stabilising agent was determined a few years later by Hood et al. (2002). The classic microtubule-stabilising agent, paclitaxel, is used clinically as an anticancer agent. Yeast has three tubulin isotypes. *TUB1* is the major α-tubulin gene of yeast and *TUB2* encodes β-tubulin. Both are essential for viability. The third yeast tubulin isotype gene, also α-tubulin (*TUB3*), is expressed at lower levels and is not essential for cell viability. Yeast tubulin shows about 75% homology to human brain tubulin (Barnes et al., 1992). Bode et al. (2002) showed that the tubulin targeting agents paclitaxel, colchicine, vinblastine, podophyllotoxin and cytophycin had no effect on yeast tubulin assembly *in vitro*; however, epothilone A and B were able to promote assembly of purified tubulin but concentrations up to 150 μM had little effect on yeast proliferation *in vivo*. By selective mutation of five amino acids (A19, T23, G26, N227, and Y270) in the yeast β-tubulin gene (*TUB2*) at the taxoid binding site, a functional paclitaxel binding site was constructed by Gupta et al. (2003), and this mutant tubulin could be polymerised by paclitaxel *in vitro*. Sensitivity of paclitaxel, however, in yeast *in vivo* required seven inactivations of ABC transporters in addition to these five mutations in β-tubulin (Foland et al., 2005). Amino acids in the proposed binding site for PelA (Gaitanos et al., 2004; Huzil et al., 2008; Kanakkanthara et al., 2011; Nguyen et al., 2010) are more highly conserved between yeast and humans than the taxoid binding site. We therefore hypothesised that PelA would be able to inhibit yeast growth in cells with wild type *TUB2*. The aim of the present study was to test whether yeast growth could be inhibited by PelA and to use yeast HIP and HOP microarray analyses to identify secondary targets and interactive networks of PelA in order to better understand its mode of action in cells. Previous studies have demonstrated that yeast can be used effectively as a model organism to study drug interactions in mammalian cells (Giaever et al., 2004; Lum et al., 2004; Menacho-Marquez and Murguia, 2007; Nislow and Giaever, 2007; Parsons et al., 2004, 2006).

2. Materials and methods

2.1. Materials

Peloruside A (PelA) was purified from the marine sponge *Mycale hentscheli* as previously described (West et al., 2000), dissolved in DMSO at 10 mM, and stored at −80 °C. As the supply of natural PelA was strictly limited, stocks used in this study were conserved as much as possible, and this impacted on the number of replicates that could be carried out. Paclitaxel was purchased from LC Labs (Woburn, MA) and stored at −80 °C in DMSO at 10 mM.

2.2. PelA inhibition of growth

A concentration–response relationship for PelA in the haploid wild type yeast strain (BY4741) of *Saccharomyces cerevisiae* was performed to determine the optimal concentration of PelA for the microarray. A concentration was chosen that only affected growth to a minimal extent (IC_{10}–IC_{20}). In addition, individual growth inhibition studies were initially carried out with paclitaxel on several deletion mutants that had been previously shown to have lethal interactions with either α- or β-tubulin in yeast, including *mad2Δ*, *mad3Δ*, *gim1Δ*, *gim4Δ*, *cin1Δ*, *cin4Δ*, *pac2Δ*, *mcm21Δ*, and *bem2Δ*. The three most sensitive strains were then tested with PelA. Mad2 and Mad3 are part of the spindle assembly complex, Gim1 and Gim4 are part of the prochaperone prefolding complex, Cin1 and Cin4 are involved in tubulin folding, Pac2 is involved in tubulin heterodimer formation, Mcm21 is involved in minichromosome maintenance, and Bem2 is involved in bud emergence.

2.3. Yeast deletion pool growth for microarray experiments

To make a pool of homozygous yeast deletion strains, the library (Open Biosystems) was grown on YPD agar (2% peptone, 1% yeast extract, 2% glucose, 0.012% adenine hemisulphate) containing 200 mg/L G418 antibiotic (Geneticin, Gibco, Invitrogen). Colonies were scraped from the plates, pooled and aliquoted in YPD plus 15% glycerol and stored at −80 °C at a cell concentration of 1×10^8 cells/0.2 mL. The heterozygous knockout pool (Invitrogen) was stored in YPD/15% glycerol at a cell concentration of 0.5×10^8 cells/0.5 mL. An aliquot of the pool was grown overnight at 30 °C in a Bioline shaker incubator (Edwards Instrument Company, Australia) in 10 mL synthetic complete medium (SC medium) consisting of 6.7 g Bacto-yeast nitrogen, 136 g yeast nitrogen base (without amino acids), 0.8 g monosodium glutamate, and 1.6 g amino acid mixture in a total volume of 800 mL and supplemented with 2% glucose, 25 mM HEPES, and 0.1% G418 antibiotic. In order to have at least 1000 cells in the mixed suspension for each mutant, 5×10^6 cells were diluted into 10 mL SC medium containing 2% glucose and 25 mM HEPES in the presence of 10 μM PelA or 0.1% DMSO (control). After 15 h (~10 generations), cells were re-diluted to 5×10^6 cells/10 mL and re-treated with PelA or DMSO for an additional 15 h (~20 generations total).

2.4. Genomic DNA purification

Genomic DNA was purified from a 1.5 mL aliquot of the treated and control cultures using a Master Pure™ yeast DNA purification kit (Epicentre Biotechnologies, Global Science & Technology, NZ), following the manufacturer's instructions, followed by RNA degradation by addition of 1 μL of RNase A (5 μg/μL) and incubation at 37 °C for 30 min. The DNA was purified by phenol–chloroform extraction, ethanol precipitated, and dissolved in 35 μL TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA was quantified using Hoechst 33258 dye (1 mg/mL) (DNA Quantification kit DNA-QF, Sigma-Aldrich) following the manufacturer's instructions. Fluorescence was measured in a SpectraMax Gemini plate reader (Molecular Dynamics, Sunnyvale, CA) at 360 nm excitation and 460 nm emission wavelengths. A typical DNA concentration of 300 ng/5 μL was obtained, and DNA aliquots were adjusted to 25 ng/μL.

2.5. Barcode amplification and Cy3/5 dye labelling by PCR

For each sample, Up tags and DN tags were PCR amplified in separate reactions. DMSO-treated controls were amplified with one primer labelled with the fluorescent dye Cy3, and PelA-treated samples were amplified with a Cy5 labelled primer. The UP tag was amplified using primers Up1 (5′-GATGTCCACGAGGTCTCT) and Up2-Cy5 or Cy3 (5′-GTCGACCTGCAGCGTACG). The DN tag was amplified by primers DN1 (5′-CGGTGTCGGTCTCGTAG) and DN2-Cy3 or Cy3 (5′-CGAGCTCGAATTCATCGAT). PCR Master Mix was prepared in a DNA-free laminar flow hood. The composition of the PCR Master Mix was (in a total of 51.2 μL): 10× platinum taq buffer (6 μL, 1× final concentration), 50 mM $MgCl_2$ (1.8 μL, 1.5 mM final), 10 mM each of dNTPs (1.2 μL, 0.2 mM final), 5 U/μL platinum taq (0.2 μL, 1 U final), and distilled water (42 μL). In PCR tubes, the 51.2 μL of PCR Master Mix was mixed with 2.4 μL labelled primer (25 μM) and 2.4 μL unlabelled primer (25 μM). Final concentrations of primers were 1 μM. To all reactions, 4 μL of 25 ng/μL DNA was added, except for negative controls in which dH_2O was added instead. The final volume of each PCR reaction was 60 μL. PCR was carried out on a T-Gradient PCR machine (Biometra®,