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I am developing a novel cell-based small-molecule screening approach that can identify inhibitors of any non-essential protein function through a surrogate synthetic lethal phenotype in the baker's yeast, *Saccharomyces cerevisiae*. Synthetic lethality (SL) is a form of genetic enhancement in which two mutations are lethal in combination, but the corresponding individual mutants are viable. Thus, a sensitized yeast strain carrying a mutation that is synthetic lethal with a gene of interest will be inviable in the presence of a chemical inhibitor of the target protein. Systematic genome-wide genetic screens can simultaneously determine all the synthetic lethal genetic interactions for a given gene deletion in yeast. I will adopt this strategy to determine the SL partners for the yeast genes *SCH9*, the yeast homologue of the human oncogene AKT, and *TEP1*, the yeast equivalent of the human tumor suppressor PTEN. Selected confirmed synthetic lethal mutants will be used as sensitized strains to screen a commercial small-molecule library for inhibitors of the corresponding proteins. I have developed a yeast-based high-throughput screening platform to screen the Maybridge small-molecule library. Compounds derived from the initial chemical genetic screen will be validated biochemically and, ultimately, tested on mammalian cells for activity against the human homologues.

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Introduction

Project summary:

My fellowship project is focused on development of a novel cell-based small-molecule screening approach that can identify inhibitors of any non-essential protein function through a surrogate synthetic lethal phenotype in the baker's yeast, *Saccharomyces cerevisiae*. Synthetic lethality (SL) is a form of genetic enhancement in which two mutations are lethal in combination, but the corresponding individual mutants are viable. Thus, a sensitized yeast strain carrying a mutation that is synthetic lethal with a gene of interest will be inviable in the presence of a chemical inhibitor of the target protein. Systematic genome-wide genetic screens have been developed to simultaneously determine all the synthetic lethal genetic interactions for a given gene deletion in yeast. I proposed to adopt this strategy to determine the SL partners for the yeast genes *SCH9*, the closest yeast homologue to the human oncogene AKT, and *TEP1*, the yeast equivalent of the human tumor suppressor PTEN. Selected confirmed synthetic lethal mutants will then be used as sensitized strains to screen a commercial small-molecule library for inhibitors of the corresponding proteins. Screening of the 50,000 compound Maybridge small-molecule library requires concurrent development of a yeast-based high-throughput screening platform. Compounds derived from the initial chemical genetic screen will be validated biochemically and, ultimately, tested on mammalian cells for activity against the human homologues. To achieve this, four main aims will be pursued:

Aim 1. High throughput infrastructure development. In order to maximize the sensitivity of yeast to the compounds I will develop yeast strains that are crippled for the pleiotropic drug resistance (PDR) network through deletion of a gene or genes that confer maximum sensitivity. I will perform synthetic genetic array (SGA) analysis on the candidate genes to minimize the potential overlap with target synthetic lethal genes in the sensitized strains. I will develop humanized versions of *SCH9* and *TEP1* to insert into the corresponding deletion mutants for testing of compounds in the context of the human gene. I will develop and optimize a screening format, either liquid or solid-phase, for testing the compounds. In addition I will develop and test a robotic screening platform for conducting the screen. I will optimize the growth and media conditions for maximum dynamic range in our scoring system. Finally, I will perform a wildtype screen against the Maybridge library to determine growth inhibition for each compound as a baseline for comparison with the sensitized strains.

Aim 2. Development of the synthetic lethal chemogenetic (SLC) screen. I will perform SGA on *sch9Δ* and *tep1Δ* strains to identify candidate synthetic lethal genes for sensitized strains. Interactions will be confirmed with tetrad analysis. In addition, I will perform SGA analysis on selected synthetic lethal candidates in order to select sensitized strains that have a unique genetic fingerprint for the screen. I will move the sensitized strains into the PDR-defective parental background for the screen. I will conduct pilot-scale screens against the 1998 compound NCI diversity set. I will then screen the sensitized strain against the Maybridge small-molecule library for chemical synthetic lethal interactions that are unique to the sensitized strain. Subtractive comparison with the data from the wildtype PDR screen will reveal any candidate inhibitory compounds. I will confirm any hits with a dose response assay of the sensitized strain vs. the

parental PDR strain. I will iteratively subscreen additional sensitized strains against a miniarray of "hit" compounds to eliminate non-specific chemical interactions.

Aim 3. Mechanism of Action. I will characterize the compound hits for activity against *Sch9* by sizing with a Coulter channelizer or with in vitro Sch9 kinase assays. I will screen candidate compounds against the deletion set for chemical synthetic lethality either by pinning or by competitive growth in liquid media followed by analysis using DNA microarrays in order to infer the pathway that is being inhibited. I will screen the compound mini-array of hits systematically for synergistic activity against wildtype and sensitized strains.

Aim 4. Survey compounds against human breast cancer cell lines. I will test the most promising hits for growth inhibition against a panel of breast cancer cell lines. I will perform dose-response assays to test growth inhibiton or apoptosis. Positive compounds will be tested for their effect on known targets of the human proteins such as a phosphorylation of BAD or dephosphorylation of PIP3.

Body

Progress

I am developing a cell-based high-throughput small-molecule screen with a genetic readout using the yeast, *Saccharomyces cerevisiae.* Yeast have evolved a pleiotropic drug resistance (PDR) network that is rapidly upregulated in the presence of potential toxins. This network consists of a number of ABC transporters similar to P-glycoprotein in the human multiple drug resistance (MDR) network. The PDR network is controlled by a pair of transcription factors, *Pdr1* and *Pdr3*. In order to maximize the bioactivity of the compound library, I have constructed a drug sensitive (DS) parental strain in which PDR activity is compromised. I tested yeast mutants missing components of the PDR network for increased sensitivity to a number of known compounds such as the pan-kinase inhibitor, staurosporine, and the topoisomerase inhibitor, camptothecin. In addition to mutants lacking the above mentioned transcription factors, I tested mutants lacking structural components of the network such as *pdr5Δ, snq2Δ, yor1Δ* as well as *erg6Δ*, a mutant defective for ergosterol biosynthesis that increases the permeability of yeast to small-molecules. Mutations were tested singly and pairwise in a dose-response assay. The double mutant *pdr1Δ::NAT pdr3Δ::URA3* increased sensitivity to a variety of tested compounds approximately five-fold over wildtype strains with no observable effect on growth rate and was selected as the parental DS strain (Figure 1).

I performed SGA analysis on the double mutant DS strain to determine the potential genetic interference with mutants selected as sensitized strains. The analysis revealed fewer than a dozen SL interactions, indicating that there was about a 1/400 chance that a random non essential mutant would be synthetic lethal with the DS strain.

Media pH may be as significant as strain sensitivity in enhancing bioactivity of compounds in living yeast. The literature suggested that camptothecin was only effective in media buffered with 25mM HEPES, pH 7.2 (Reid, Kauh et al. 1997). This turned out to be true for staurosporine and, as I will address in a later section, many of the compounds in the

Maybridge library as well. Buffering with 25mM HEPES increases the media pH to 6.2 which is within the limits for maximal yeast growth.

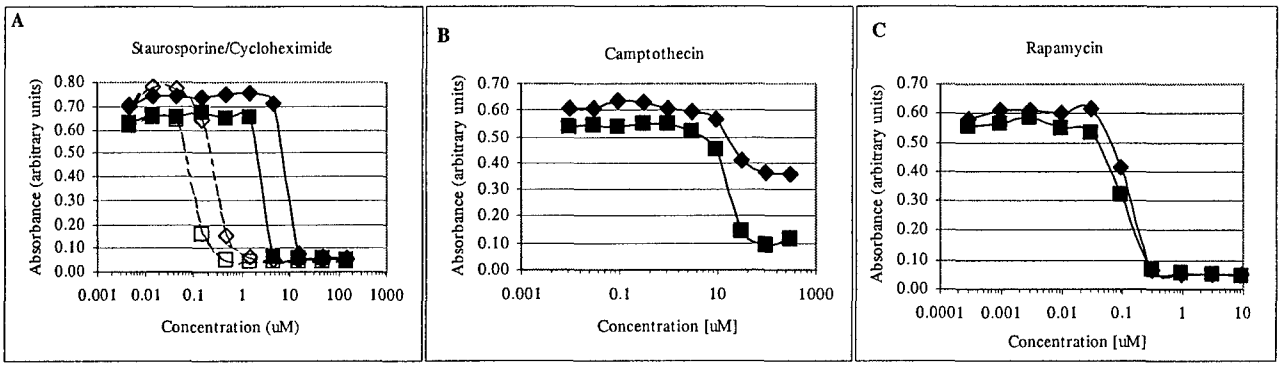

Figure 1. Drug-sensitive (DS) yeast mutants display a range of responses to small molecules. The *pdr1Δ pdr3Δ* DS mutant (squares) was compared to a wildtype strain (diamonds) in a 0.5 log dose response assay. A) The DS strain shows ≈5-fold increased sensitivity to both staurosporine (filled symbols) and cycloheximide (open symbols). This sensitivity increase was independent of compound potency. B) The DS mutant shows ≈ twofold increased response (growth inhibition) over wildtype to camptothecin. C) Rapamycin is representative of a subset of compounds that have PDR-independent activity.

Much of the initial performance period was spent developing the assay format for the high-throughput screen. I began development of a solid-phase halo assay. In this format, the yeast are grown to saturation and diluted into soft agar at high concentration, then poured as a thin film on top of a 2% agar substrate in omnitrays used for SGA analysis. The agar is allowed to set at 4°C overnight to prevent cell division and the plates are pinned robotically with ≈100nL of compound using a 96-pin floating head. The robots allow for automated washing and iterative pinning of 20 plates/run for each robot for a maximum throughput of 850 compounds/hour. The halo assay provides additional information that is lost in a liquid growth competition format. Because the yeast are restrained spatially, halo diameter is proportional to compound potency. Turbidity of the halo is indicative of growth inhibition rather than toxicity. Two factors limited the suitability of this assay for high-throughput screening. One limiting factor was the difficulty in pouring the yeast layer in a uniform thickness at a uniform density. Differences in substrate thickness and cell density translated into large changes in halo parameters. In addition we were not able to adapt our automated scoring system from the SGA screen to the halo plates because of the physical limitations of the format. Thus, the scoring would have to be performed by eye and would be entirely qualitative. Combined with the variable nature of the halo layer, the manual scoring made this format untenable.

I then established a collaboration with Eric Brown, who has built an industry-scale high-throughput screening facility at McMaster University in Hamilton, Ontario. The facility also has a master copy of the Maybridge library. I was given access to a Biomek FX work station and received training on programming of the Biomek as well as an introduction to Spotfire chemical database software at the HTS facility. I developed a liquid growth inhibition assay. Scoring was quantitated by measuring optical density at 600nm using a dedicated plate reader and results were calculated as residual growth compared to internal positive and negative controls on each plate. The assay required optimization of variables such as growth conditions, initial yeast density,

endpoint timing, media pH, and DMSO tolerance. The optimization assays were conducted in pilot screens using the Maybridge HitsKit, a 1000 compound mini-array that surveys the chemical space of the full library. I chose to use this sublibrary for optimization instead of the NCI diversity set as outlined in my proposal because it allowed us to interrogate the conditions using the actual compounds in the screen. Fully 80% of the compounds that caused growth inhibition in the buffered media were inactive in synthetic complete media alone, while only a subset of compounds killed yeast equally in both conditions (Figure 2) After optimization, we calculated a Z' score (a statistical measure of the assay's dynamic range) of 0.55-0.61, indicating that our assay was robust enough to reliably differentiate hits from noise.

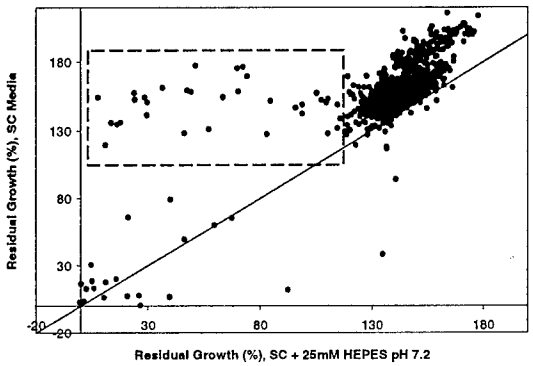

Figure 2. Compound biocativity is dependent on media pH. Replicate plot of relative yeast growth compared to DMSO control in unbuffered synthetic complete (SC) media (y-axis) versus SC buffered with 25mM HEPES pH 7.2 (x-axis). Dashed box indicates compounds that were within 3SD of DMSO control growth in SC, but fell below 3SD residual growth in buffered media. Of the total bioactives, 80.85% (38/47) were only active in buffered media. Cytotoxic compounds (scoring below 30% activity in both screens) were excluded from the calculations.

I commenced SGA analysis of *sch9Δ* in order to construct the sensitized strains for the synthetic lethal chemogenetic (SLC) screen. Null mutants of *SCH9* have a severe size and growth phenotype and rapidly accumulate suppressor mutations that complement the loss of *Sch9*. To counteract this effect, I carried out the screen using a *sch9Δ* mutant that was complemented with *SCH9* on a URA3-marked plasmid that could be selected against to unveil the deletion in the final pinning step by addition of 5'fluoro-orotic acid (5'FOA) to the plates. Unfortunately, the final pinning step required a slow growing strain to repopulate from a small number of initial cells. This caused wide variability in final colony size and increased the false positive rate to over 95%. The initial screen produced a single confirmed synthetic lethal partner out of 43 query genes.

To overcome this problem, we developed an analogue-sensitive kinase allele (ASKA) of *SCH9* using the method of Shokat (Bishop, Ubersax et al. 2000). In this method, a kinase is mutated at a conserved location in the active site that allows it to accept a nucleoside analogue with a bulky residue that cannot fit in the wildtype protein, thus creating an allele with its own highly-specific inhibitor. With this ASKA mutant, I could perform chemical SGA in which the query gene is shut off by addition of a chemical in the final pinning step. The resulting *sch9-AS* mutant was a hypomorph with size and growth characteristics approximately midway between the null mutant and *SCH9* (Jorgensen, Rupes et al. 2004). Addition of the analogue 1-naphthylmethyl PP1 phenocopied the null mutant's size and growth defects, but had no effect on *SCH9* cells at the concentration used in the screen (Jorgensen, Rupes et al. 2004). Comparison using our automated scoring algorithm showed that the chemical SGA eliminated both false negative and false positive interactions. Interactions discovered using chemical SGA were confirmed genetically by mating candidates back to *sch9Δ* and performing tetrad dissection. The

chemical SGA revealed 8 new SL interactions, including a previously published SL gene (denoted with an asterisk), validating the method (Table 1).

Table 1. SCH9 SGA Analysis: Confirmed SCH9 Synthetic Lethal Genes

ORF	YNL067W	YGR085C	YER056C-A	YNL084C	YDL035C	YDL115C	YBR255W	YOL138C
NAME	RPL9B	RPL11B	RPL34A	END3	GPR1*	IWR1		

The *sch9-AS* allele provides an excellent proof-of-principle allele to validate the SLC methodology. To test the proof-of-principle, confirmed *sch9Δ* SL deletions were moved into a strain containing the *sch9-AS* allele integrated at the *SCH9* locus. A dose response with 1NM-PP1 showed that there was an 80% reduction in residual growth between the double mutants and *sch9-AS* alone (Figure 3). Interestingly, even synthetic fitness interactions could be differentiated from the AS allele in this assay. The analogue 1NM-PP1 had no effect on SL mutants containing *SCH9* at the maximal concentration, demonstrating the specificity of the SLC interactions. As an additional negative control, the analogue 1-naphthyl PP1 showed no effect on either the AS allele or the double mutants.

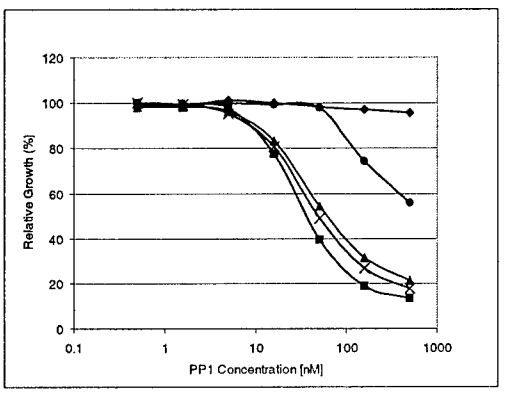

Figure 3. Proof-of-principle using the *sch9-AS* allele. *SCH9* synthetic lethal deletion mutants were moved into the background strain containing the *sch9-AS* allele. *Sch9-AS* double mutants containing *rpl9bΔ* (squares), *rpl11bΔ* (triangles), *gpr1Δ* (crosses), *and the synthetic fitness mutant, yol138cΔ* (circles) were tested in a 0.5log dose response assay along with the *sch9-AS* strain (diamonds). Relative growth was measured against isogenic strains treated with DMSO. Optical density of the cultures was measured at 20 hours post inoculation.

I undertook a full-scale screen with the Maybridge small molecule library at the HTS facility at McMaster University. I first moved the *sch9Δ* SL strains into the DS background. I screened the parental DS strain against the library in duplicate at 50 micromolar compound concentration. I chose to test *rpl9bΔ* as the first sensitized strain because it exhibited the strongest genetic interaction with *sch9Δ* and I hypothesized it would give increased specificity since its overlapping SL interactions would be reduced by the presence of a complementing allele. The sensitized mutant was screened in duplicate as well and showed excellent replicability with an activity profile similar to the DS strain. Using Spotfire database software, I sorted the results for compounds that were within 3SD of DMSO control growth in the DS screen, but were ≥5SD below DMSO growth in the sensitized strain. Analysis using these stringent criteria produced 77 potential hits (Figure 4). These 77 were cherry picked from the library and tested using a differential dose response with DS and sensitized strains. Twenty-one compounds exhibited chemical synthetic lethality at the screening concentration.

Currently, I am working to acquire milligram quantities of the 21 compounds for testing against other sensitized strains and in secondary, phenotype screens. Our institute has a DIM4 robot from CRS that is just coming online and will allow us to pin nanoliter quantities of compound into liquid cultures, a hybrid of the two assays I optimized in this performance period. I am beginning optimization screens to translate our method to this new resource so we can begin

screening at Mount Sinai. SGA analysis of genes that are SL with *sch9Δ* is ongoing as well as the initial *tep1Δ* SGA analysis.

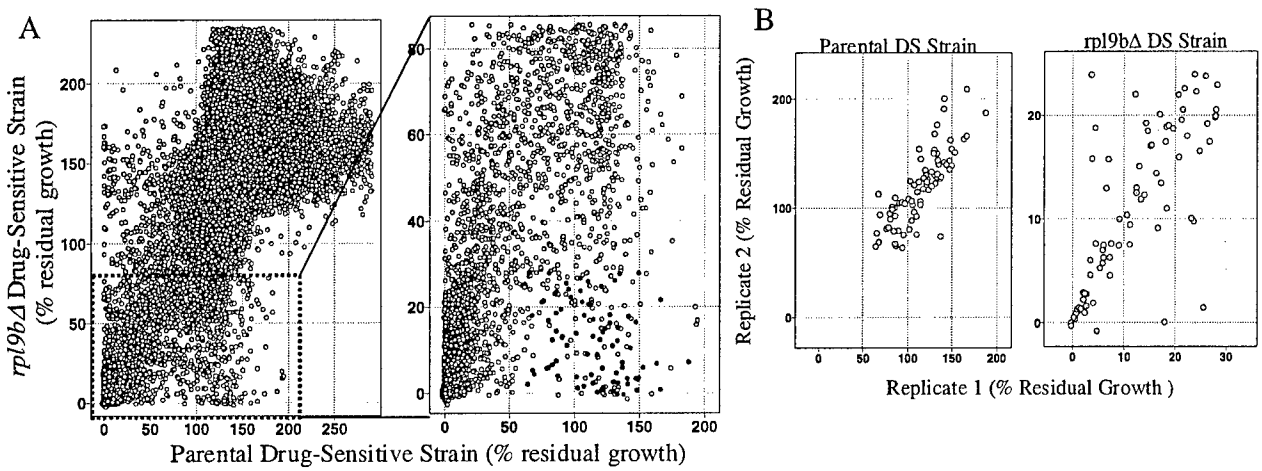

Figure 4. Full-scale differential SLC screen of *rpl9bΔ* DS strain versus parental DS mutant against the Maybridge small molecule library. A) Replicate plot of parental DS growth (x-axis) versus *rpl9bΔ* DS (y-axis). Residual growth (%) is in comparison to internal negative (DMSO) and positive (cycloheximide [CHX] 50uM) on each plate (sample-CHX/DMSO-CHX)*100. Inset magnifies the region of differential growth (dashed box). Potential hits identified by Spotfire are denoted by black filled circles. B) Replicate plot of individual hits against each strain background. Residual growth of the 77 hit compounds plotted as individual replicates for the parental strain (left panel) or the *rpl9bΔ* sensitized strain (right panel). Note the scale difference between the two panels.

List of Key Accomplishments:

- Developed high-throughput screening platform for cell-based yeast assay.
- Developed chemical SGA analysis using analogue sensitive kinase allele (*sch9-AS*).
- Demonstrated the validity of chemical SGA.
- Determined synthetic lethal genetic interactions with *sch9Δ* using chemical SGA.
- Demonstrated SLC proof-of-principle using *sch9-AS*.
- Conducted large-scale high-throughput SLC screen of parental DS and sensitized *rpl9bΔ*strains..

Reportable outcomes

Keystone Symposium: New Advances in Drug Discovery, Keystone, CO March 21-26, 2004
Poster (104): "The Synthetic Lethal Trap: a novel genetic assay to probe for specific chemical inhibitors of non-essential proteins using HTS" David S. Bellows, Paul Jorgensen and Mike Tyers. The Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto

Travel Scholarship: Keystone Symposium. New Advances in Drug Discovery, Keystone, CO March 21-26, 2004

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Jorgensen, P., I. Rupes, et al. (2004). "A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size." Genes Dev. **18**(20): 2491-2505.
Reid, R. J. D., E. A. Kauh, et al. (1997). "Camptothecin Sensitivity Is Mediated by the Pleiotropic Drug Resistance Network in Yeast." J. Biol. Chem. **272**(18): 12091-12099.