

Chemical Biology: Systematic Exploration of Drug Synergy in Haploid *Saccharomyces cerevisiae*

J. Fagan¹; V. Gelev, PhD²; S. Lokey, PhD³

¹[SURF Fellow, Berea College Biology and Chemistry Undergraduate Student], ²[University of California – Santa Cruz and Harvard University Chemistry Post Doctoral Fellow], ³[University of California – Santa Cruz Assistant Professor of Chemistry]

JSURF – Journal of the Summer Undergraduate Research Fellowship
Sponsored by the Department of Chemistry at the University of California- Santa Cruz (UCSC) and
The National Science Foundation (NSF)

Abstract:

A collection of 15,136 drug-like molecules were systematically analyzed for their antifungal effectiveness. The molecules were tested with a haploid *Saccharomyces cerevisiae* (yeast) liquid culture assay and the absorbencies of the yeast solution related to growth and compared to a standard control. Of the 15,136 compounds tested, molecule C239-0032 (ChemDiv Code name) was found to be the most significant antifungal, confirming a previous agar assay study by V. Gelev. The liquid culture assay was also performed with the 15,136 compounds in combination with a sub-lethal dose of C239-0032 background to search for molecules which act as an antifungal synergistically, in combination or produce a synthetically lethal affect. Synergistic, combination, and synthetically lethal type therapies might provide useful benefits such as decreased fungi mutation rates and decreased dosage which could minimize potential side effects. The results of the C239 combination assay provided a useful list of potential synergistic, combination, or synthetically lethal molecules which will further be confirmed this upcoming year. Isobolograms and drug target assays will be performed on molecules of interest in attempt to determine the target genes in yeast affected by C239-0032 and the potential molecules with combination, synergistic, and/or synthetically lethal affects. Resulting useful molecules will likely be tested on *Candida* and other strains of yeast directly affecting humans. The study will continue throughout the 2006-2007 year.

Introduction:

Chemical biology, a more recent subdivision of chemistry and recognized by the American Chemical Society (ACS), is becoming increasingly important in other subfields and practical applications. Chemical biology is often confused with biochemistry. Common accepted definitions of biochemistry include the study of the chemistry of life, the study of the chemical substances and vital processes occurring in living organisms, and the chemical composition of a particular living system or biological substance. Chemical biology differs from biochemistry in that it aims to answer many questions arising from biochemistry by using tools developed within synthetic chemistry. Chemical biologists attempt to regulate any biological process with specific small molecule regulators and use non-DNA, non-RNA, and non-protein like molecules which can regulate or be regulated by DNA, RNA, and protein.

One such application of chemical biology is in relationship to medicinal chemistry, in particular in the search of novel antifungal medications. Antifungal medications include drugs used to treat a range of fungal infections. Common fungal

infections include athlete's foot, *candidiasis* (yeast infections), and ringworm. Antifungal medications can be a particular challenge due to the nature of fungi.

Bacteria are prokaryotic organisms, whereas human cells are eukaryotic. The differences between eukaryotic and prokaryotic organisms provide an easier opportunity for antibiotics to selectively stop bacteria growth to end infections with little harm to human cells. However, fungi also have eukaryotic cells like humans. The similarities in the cells make it more difficult to find drugs which will selectively kill fungi cells. As a result, antifungal drugs can cause increased side effects, which, in severe instances, could lead to death.

The purpose of the study was to use chemical biology methodology, in the use of small drug-like molecules, in an attempt to overcome some of the challenges associated with the discovery of new antifungal drugs. The target organism of the study included wild type haploid *Saccharomyces cerevisiae*, otherwise known as bakers yeast. Yeast is both safe and cost effective. It serves as a model organism which is significantly studied in the scientific community. Homology of the genetic fingerprints of yeast strains can allow future practical application to other strains of yeast and fungi which directly affect human health.

The 15,136 small molecules used in this study are from a library supplied from ChemDiv and are drug-like for reasons explained by Christopher A. Lipinski (1). Lipinski identified features of molecules which were commonly found in orally active drugs (1). These features are known as Lipinski's rule of five. They are a general indicator of whether a drug can be bioactive (1). The rules include: < 5 hydrogen bond donors, < or = 10 hydrogen bond acceptors, less than 500 MW, and a LogP < 5 (1). From V. Gelev testing the 15,136 drug like molecules on yeast growth with an agar assay, one of the molecules, C239-0032, became of particular interest (Figure 1). C239-0032 showed signs of being a powerful antifungal. To confirm the result of the small molecule, a more sensitive liquid culture assay was used in this study.

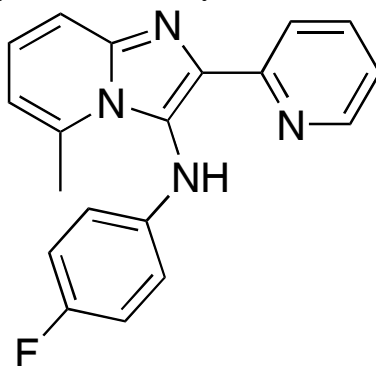


Figure 1. ChemDiv C239-0032 Small Molecule Chemical Structure.

Since fungi are similar to human cells, C239-0032 and other potential drugs can have increased side effects. As a result, combination therapy can provide a useful tool in solving this problem. A combination of drugs adds two effects for drug therapy, in simple terms, 1 drug + 1 drug = the effect of 2 drugs. However, if a combination proves to be synergistic, 1 drug + 1 drug = the effect of 3+ drugs. Combinations or a synergistic combination will decrease the amount of one particular drug an individual must receive, which would hopefully decrease patient side effects.

Besides side effects, mutation rates in fungi and bacteria can create difficulties in new therapeutic remedies. Also, one particular drug target might not be efficient in killing an organism. Biochemical pathways are complex in nature. In yeast, if a drug compound harms a protein or gene, many times, the yeast can continue to function through an interconnected interface of biochemical relationships (Figure 2).

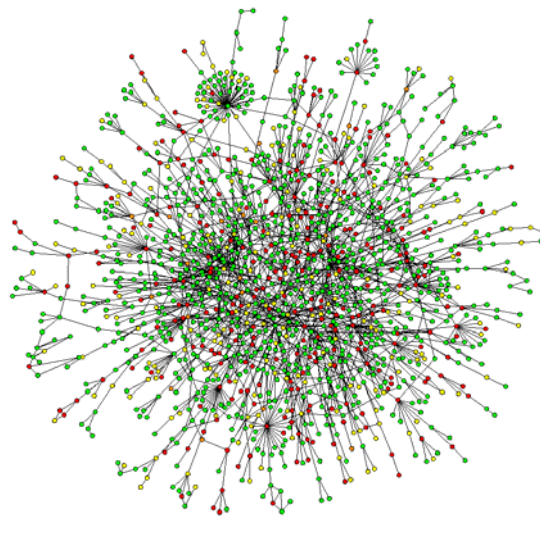


Figure 2. Map of Protein-Protein Interactions in Yeast. Red are lethal genes, green are non-lethal genes, orange are slow growing, and yellow are unknown (2).

Combinations can also have a synthetically lethal affect. Synthetic lethality creates a lethal event that would otherwise not occur unless two events took place simultaneously (Figure 3). Drug combinations can lead to drug cocktails which might not have otherwise been explored with individual molecules.

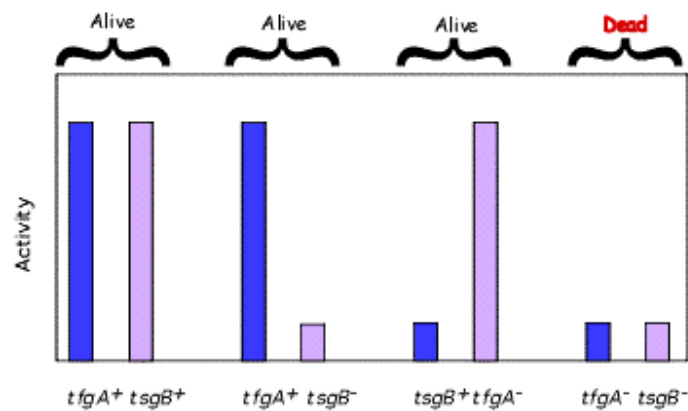


Figure 3. Explanation of Synthetic Lethality. The absence of gene A or B does not cause a lethal effect individually, but if both are absent the result is fatal, gene A and B are synthetically lethal (3).

The study took C239-0032, a potential antifungal, and retested the compound and entire compound library in a liquid culture assay for confirmation. A sub-lethal dose of

C230-0032 was determined previously by V.Gleve. This sub-lethal dose was used to test C239-0032 in combination with the compound library. These combinations were hoped to create synergistic, direct combination effects, and/or synthetically lethal events. Due to the drug-like nature of the molecules, it is hoped they could be potential drugs which could provide beneficial applications to pharmacology.

Methodology:

Yeast liquid culture assay techniques were used to analyze a 15,136 compound collection from ChemDiv. ChemDiv, a scientific company based in Russia, supplied the library of drug like molecules. The liquid yeast culture techniques were developed by researchers at UCSC. The particular protocol used for this study is as follows:

Media and Yeast Culture:

YPD media (10g/L yeast extract, 20g/L peptone, and 20g/L dextrose) was made and autoclaved for 25min on the fluid setting. YPD (5mL) and a colony of haploid *Saccharomyces cerevisiae* were combined in an aerobic exchange test tube and placed in a spinner overnight at 30 degrees Celsius to grow. NaOH/HCl was added to the remaining YPD to pH 7.0.

Assay Condition (with C239 background = plus):

The yeast culture was diluted 1/500 with YPD media. A solution of 10mg/mL of C239 in DMSO was diluted in the mixture 3ug/mL of C239 in YPD media. The amount of C239 was previously determined by Dr. Vladimir Geleve as a sub-lethal dose by conducting a concentration array test.

Assay Condition (without C239 = minus):

The yeast culture was diluted 1/500 with YPD media. DMSO was diluted in the mixture. The volume added of DMSO equaled the volume added of C239 in DMSO added to the plus assay condition.

Assay Preparation and Analysis:

Assay trays of 384 well plates were used. A multidrop machine was used to place 20uL of assay solution into each well. Plates were labeled either plus or minus for the relative assay condition. The ChemDiv Library of 43 compound plates containing 384 wells, the last 32 containing control wells for a total of 15,136 compounds was used. A VP930B 384 needle robot was used to distribute 2uL of compounds from the 384 well compound plates to the 384 well assay trays. Needles were washed in-between cycles with DMSO and methanol baths. Assay trays were left on a shaker overnight and measured the next morning on an absorbance plate reader. Through WALLAC work station, before reading, each tray was agitated for 5 minutes and then an absorbance immediately read. Data was exported to excel for analysis. The plus and minus corresponding trays were prepared and analyzed simultaneously to control differences in the yeast culture and temperature.

Confirmation Analysis:

Resulting hits in the analysis of C239 background to the control were retested. An agar assay was performed. Agar 0.75g/100mL was made and 25mL of agar along with 400uL of yeast culture and 23uL of 10mg/mL C239 in DMSO were added to an assay tray. Blot paper, about 3mm in diameter, was added to the agar in 1/2inch spaces and 5uL was dispensed onto the blot paper. Concurrently, a control without C239 was run in parallel,

instead with 23uL of DMSO. After the yeast was allowed to grow over night, visual observations were made and recorded.

Results:

The plate absorbance data was standardized by finding the average and standard deviation of the 32 control wells of each 384 well assay plate. Two comparisons were made for each plate. The fraction difference from the average control absorbance was determined for each individual absorbance as follows: (well absorbance – control average absorbance)/ (control average absorbance). The difference from the average control was also found in standard deviations as follows: (well absorbance – control average absorbance)/ (standard deviation of control average absorbance). While both graphs are the same, they provide different unit value comparisons. For each set of plus and minus assay trays for each compound plate, the minus and plus absorbencies were graphed simultaneously along with the difference.

Absorbencies were directly related to growth. Yeast growth clouds the media. A lower absorbance was related to a lower growth rate and the added compound was considered a more effective antifungal. Of the 43 compound plates, plate 10, well C17, showed the lowest absorbance compared to the entire library. The absorbance was over 25 standard deviations away from the average (Figure 3). The compound was code C239-0032 from the ChemDiv library. Other notable molecules were observed and recorded if above 10 standard deviations from the control (Table 1).

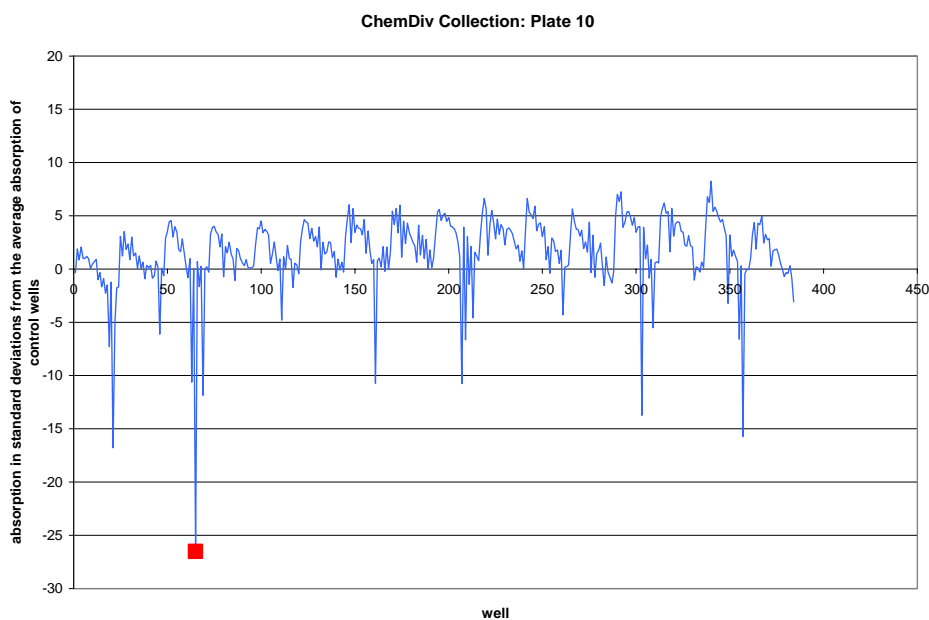


Figure 4. Liquid Culture Results Referenced to Control – Determination of C239 as a major hit, Plate 10 containing 384 wells of the ChemDiv Collection.

Table 1. Potential Antifungal Compounds: Absorbencies with a standard deviation of >10 standard deviations from the control average.

Plate 42 – multiple hits
Plate 41 – 3 or 4 hits
Plate 40 – 1 hit
Plate 33 – M3 looks like a regular hit
Plate 13 – 4 or so regular hits
Plate 10 – multiple hits – C239-0032 the largest of entire library
Plate 6 – multiple hits, one large hit
Plate 5 – several hits
Plate 3 – multiple hits
Plate 1 – multiple hits

When looking at the plate comparisons for mixtures with C239, a visual assessment of difference was used to determine potential combinations of interest. Assay plate wells which showed a decreased absorbance from the minus to the plus were observed (Figure 4). Peaks showing a difference larger than 5 standard deviations from the average were recorded (Table 2).

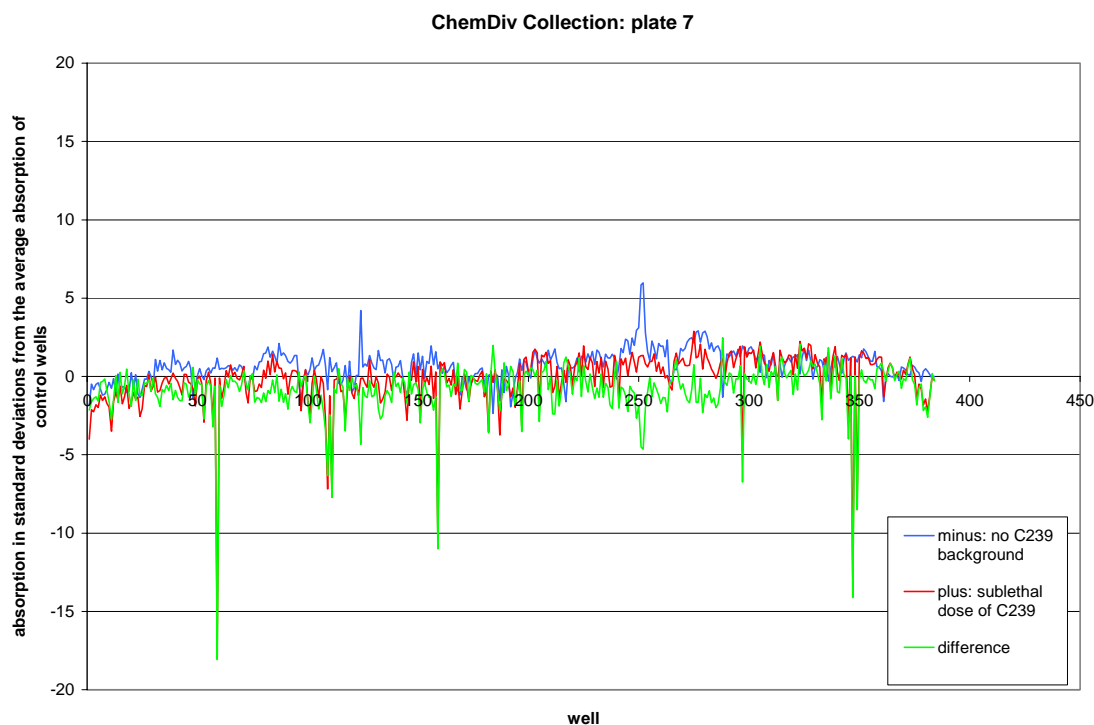


Figure 5. Liquid Culture Results Referenced to Control with C239 Background – Determination of Combination Effects, Plate 7 containing 384 wells of the ChemDiv Collection. Potential Target Combinations show a large difference (Green).

Table 2. Potential C239 Combinations: Absorbencies with a difference of >5 standard deviations from the control average.

Plate 39 – A11
Plate 37 – M01, N05
Plate 36 – B13, H19
Plate 35 – P07
Plate 33 – B20, D2, L14
Plate 32 – A7, A16, A21, D10, G1, H7, G2, C19
Plate 23 – P20
Plate 22 – A16, O02
Plate 21 – F7
Plate 10 – O13
Plate 7 – C12, E13, E15, G15, M09, O11, O13
Plate 4 – L13
Plate 2 – B4, D3, D4, H14, K4, K7, K8, L3, L7, L11, L14, N8, N11

When combination molecules of interest (Table 2) were confirmed in an agar assay, no visual differences could be determined. The control agar plate without C239 looked identical to the agar plate with a C239 background. Decreased yeast growth around any of the compounds was not visible.

Discussion:

C239-0032 showed the largest antifungal properties, as confirmed by the liquid culture assay. A list of potential drug combinations resulted. These drug combinations will be further studied. When attempting to confirm combinations in an agar assay, no observable differences were noted. The agar assay seems to be less sensitive to the compounds than the liquid culture assay. The confirmation assays will be continued in a liquid culture form.

The C239 compound will be studied to try to determine drug targets. Through the determination of gene targets, we can better understand the mode of action of C239 and its potential for human use as an antifungal. Some preliminary results have been promising to determine gene targets affected by C239 (Figure 6). Collaborators at Stanford University used C239 to screen (~1200) essential genes as heterozygotes and (~4800) non-essential genes as homozygotes (4). Preliminary results showed genes IMP2', PHO86, MGA2, YPK1, ZAP1, and RCY1 all show sensitivity to C239 (4). These genes generally are involved in DNA repair (4).

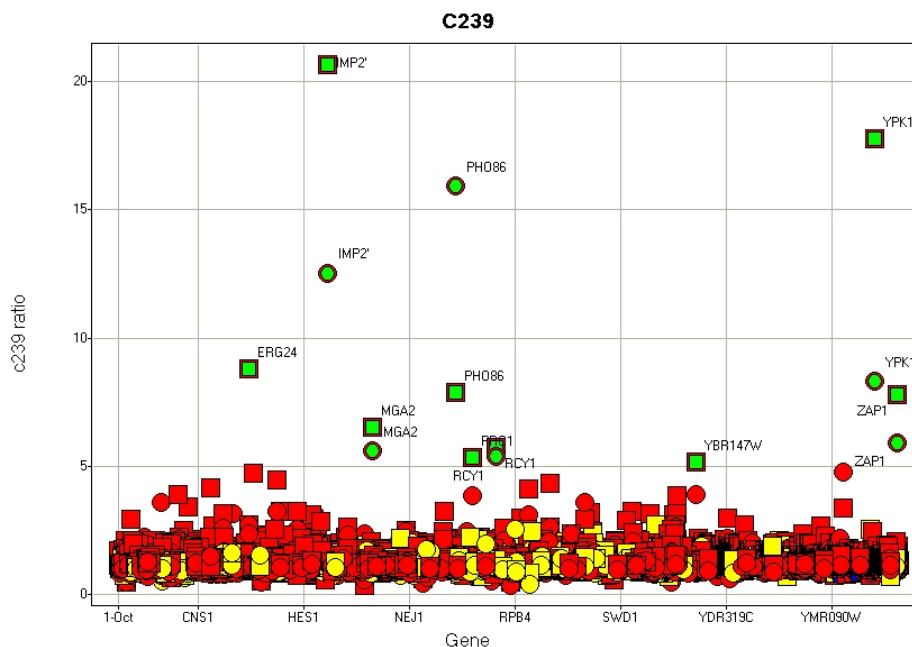


Figure 6. Preliminary Results of C239 Gene Target Affects (4)

Other data suggests that C239 might be involved in cell biosyntheses (5). Future projects seek to learn more about C239 and its affects as an antifungal. A more detailed understanding of how C239 affects genes and proteins is sought. Other information is also sought on how C239 works in combinations with the determined target list. These effects can be further confirmed and study with isobolograms and concentration dependent assays. C239 and C239 combinations show promising results. These results could lead to novel antifungal remedies with practical human application.

Acknowledgements:

Special thanks are given to the Dr. Vladimir Gelev, Dr. Scott Lokey, and members of the Lokey Research Group for all of their guidance and support. Thanks are given to those who helped to organize and support the Summer Undergraduate Research Fellowship (SURF) program: the National Science Foundation, Dr. Phil Crews, Ms. Sharon Grof, and Ms. Nicole Hill.

Other Collaborators Working on the Project:

Robert St. Onge
 Ronald Davis
 Guri Giaever
 Stanford University
 University of California - Davis

Works Cited:

(1)C. A. Lipinski, F. Lombardo, B. W. Dominy, P. J. Feeney, *Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings*. Adv. Drug Del. Rev **23**, 3-25 (1997).

(2)Hawoong Jeong, Sean Mason, Albert-László Barabási and Zoltán N. Oltvai. *Lethality and centrality in protein networks*. Nature **411**, 41-42 (2001).

(3)Hartman, J. IV, G. Garvik, and L. Hartwell. 2001. *Principles for the buffering of genetic variation*. Science **291**: 1001-1004 (2001).

(4) Onge, Robert St. and Scott Lokey. Email Communication (7-12-2006).

(5) Kellogg, Doug and Scott Lokey. Email Communication (8-6-2006).

Works Consulted:

(1)Ben Montpetit, Ken Thorne, Irene Barrett, Kim Andrews, Ravi Jadusingh, Phil Hieter and Vivien Measday. *Genome-Wide Synthetic Lethal Screens Identify an Interaction Between the Nuclear Envelope Protein, Apq12p, and the Kinetochore in Saccharomyces cerevisiae*. Genetics **171**, 489-501 (2005).

(2)James A. Karlowsky, Daryl J. Hoban, George G. Zhanel, and Beth P. Goldstein. *In vitro interactions of anidulafungin with azole antifungals, amphotericin B and 5-fluorocytosine against Candida species*. International Journal of Antimicrobial Agents **27**, 174–177 (2006).

(3)Ronald J. Tallarida. *Drug Synergism: Its Detection and Applications*. Pharmacology and Experimental Therapeutics **298**, 865–872 (2001).