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Genome-Wide Profiling Identifies *Saccharomyces Cerevisiae* Response in PKC-SLT2 Signaling and Glycogen Metabolic Pathways to Antifungal Compound Calcofluor White

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Abstract: Fungal infection remains a major problem worldwide, yet treatment options are limited owing to the lack of effective drugs, the significant toxicity of available compounds, and the emergence of drug resistance. The low toxicity of calcofluor white (CFW) is an attractive antifungal compound for its known inhibitive effects on *trichophyton rubrum* and *candida albicans* growth. However, the efficacy of CFW is limited in most cases. In order to search for effective means to improve its efficacy, using *saccharomyces cerevisiae* as a model, we have used microarrays to examine the cell's response when treated with CFW on the genome scale. We found that both the PKC-SLT2 (i.e, protein kinase C-mitogen activated protein kinase) and the glycogen metabolic pathways are activated upon CFW treatment. These results suggest that the key components in these pathways could be targeted by other drugs to counter the cell's compensative response, thus to further substantiate the inhibitive effect of CFW on fungal growth, which may lead to treatment regimens with improved efficacy of this compound in clinical applications.
Key words: *saccharomyces cerevisiae*, antifungal, calcofluor white, gene expression, signaling pathway
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0 Introduction

It is widely recognized that fungal pathogens can have significant influences on plants and animals and can cause various illnesses in humans with many of them life threatening[1-2]. For example, superficial infections of the skin and nails by dermatophytes are the most common fungal diseases in humans and affect about 25% of the general population worldwide[3]. Even though invasive fungal infections have a lower incidence than superficial infections, invasive diseases remain a great concern because they are often associated with a high mortality rate[4]. Many topical or oral drugs have been developed to combat these ubiquitous pathogens[5-6]. However, the treatment efficacy is often limited by side effects. Since fungi are eukaryotic cells, they share many of the basic biochemical processes and signaling pathways with higher eukaryotes. Therefore, many drugs against fungi are also toxic to the host. This is one of the primary reasons why fungal infections are often difficult to control and the identification of safe and effective antifungal drugs remains a major task. Calcofluor white (CFW), extensively used in the paper industry and clinical histology over many decades[7], is also known as a broad spectrum antifungal compound. For example, CFW has already been used to treat dermatophytosis[8]. Theoretically, CFW's low toxicity to humans should make it an ideal candidate to explore for treating fungal infections[8]. However, limited efficacy has hindered CFW to be applied in clinical treatments. One of the major reasons for limited efficacy is the fungal adaptation, which can compensate for the damage or disturbance caused by CFW[9]. Therefore, if this compensative response is better understood, this may help us to identify new drugable targets where a combinatorial application of other compounds could lead to more effective treatments of fungal infection at a higher efficacy and low toxicity.

Previous studies have shown that the most clearly identified effect of CFW is its direct binding to the nascent chitin chains competing for hydrogen bonding sites. Because chitin is an essential component of the fungal cell wall, this competition leads to the compromise of the cell wall integrity, and ultimately hinders the fungal growth[10-11]. However, under this inhibition, the fungal cell activates its cell wall related pathways

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to compensate, leading to the reduced efficacy. In fact, in most cases, CFW could only reduce the growth rate but could not completely eliminate the infection. So far, it is not clear whether other pathways rather than those directly related to cell wall integrity in the cell could also be perturbed by CFW under such conditions which also contribute to the survival of the cell.

To address this question, we have used microarrays to determine genome-wide transcriptome changes upon the application of CFW using *saccharomyces cerevisiae* as a model system. As shown in numerous studies, microarrays are a cost effective and sensitive platform for quantitative measurements of transcript levels rapidly and have been successfully utilized in quantitate cellular responses to small molecule stimuli of various single cell organisms[12-13]. Using this approach, we have identified that PKC-SLT2 (i.e, protein kinase C-mitogen activated protein kinase) signaling process is indeed activated as expected. But more importantly, our results also clearly demonstrate changes in other pathways related to cell survival that could be considered as potential targets for intervention in combinatorial treatment regimens.

1 Materials and Methods

1.1 Chemicals and Stock Solutions

CFW was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in distilled water. CFW stock solutions were stored at −20°C and were protected from light exposure.

1.2 Yeast Strains, Growth Conditions and CFW Treatment

Saccharomyces cerevisiae strain S288C was cultivated in YPD (1% yeast extract, 2% Bacto peptone and 2% glucose, mass fraction) medium at 28 °C. Overnight cultures were diluted to OD_{600} = 0.05, grown to OD_{600} = 0.2 and then divided into three equal fractions, where OD_{600} is optical density (or absorbance value) of yeast at 600 nm in spectroscopy. One part served as a negative control, while the second and the third parts were added with CFW for 3 h at the final concentration of 10 and 100 mg/L, respectively. Growth curves were determined by measuring the OD_{600} value of the *saccharomyces cerevisiae* culture. For microarray experiments, cells were collected by rapid centrifugation, frozen in liquid nitrogen and kept at −80°C until use.

1.3 RNA Extraction and Microarray Analysis

The change of gene expression patterns for *saccharomyces cerevisiae* that was treated with 10 mg/L CFW for 3 h was determined with affymetrix yeast gene expression microarray. Biological repeat was also performed. Total RNA from frozen *saccharomyces cerevisiae* cells was extracted with the standard Trizol® method. The qualities of the extracted RNA were determined with gel shift assays. NanoDrop 1000 spectrophotometer was used to determine the quantity and concentration of the extracted RNA. Antisence RNA (aRNA) used for hybridization was prepared according to the affymetrix protocol in the GeneChip Expression Analysis Technical Manual. Briefly, total RNA was reverse-transcribed into first-strand complementary DNA (cDNA) and then second-strand of cDNA was synthesized, followed by *in vitro* transcription to produce sufficient amount of biotin-labeled aRNA. These labeled products were then fragmented and used for microarray hybridization according to the affymetrix protocol. The GeneChip Yeast Genome 2.0 array from affymetrix used in our experiments contains approximately 5 744 probe sets for 5 841 of the 5 845 genes present in *saccharomyces cerevisiae*. In total, 3 array experiments were performed, 1 negative control and 2 biological replicates for the 10 mg/L CFW treated group.

Statistics software "R/Bioconductor" was used to analyze the array data. The "rma" function from the "affy" package was applied to background correction, normalization and summarization. The final, normalized expression of each gene was the logarithm of its signal intensity to the base 2. To determine the reproducibility of biological replicates, we performed linear regression analysis with R^2 platform. The R^2 is 0.999 5, indicating excellent reproducibility (Fig. 1).

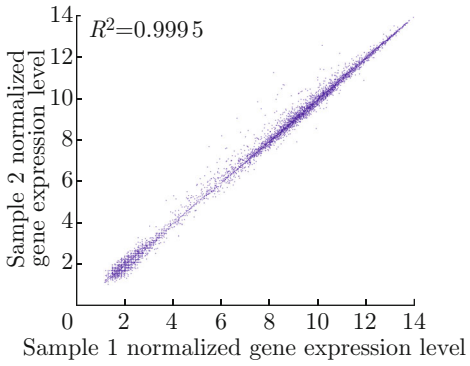


Fig. 1 Correlation analysis between two biological replicates treated with 10 mg/L CFW for 3 h

Differential expression analyses are performed for the 10 mg/L CFW treated culture against the control, using the "limma" package from the "R/Bioconductor" software. Genes with 1.5 fold change or greater between the treated and the control were determined as differentially expressed genes and the probability P-value cutoff used was 0.05.

Gene ontology (GO) analysis in the DAVID Web tool was used to perform pathway enrichment for the differentially expressed genes.

2 Results

2.1 Growth Curves of *Saccharomyces Cerevisiae* Under Different Doses of CFW

S288C was used in our experiments, and the growth curve was determined by measuring the OD_{600} value of

the *saccharomyces cerevisiae* culture (Fig. 2). In Fig. 2, under the control conditions (negative control (NC), black growth curve in Fig. 2), the growth curve shows the typical replication curve. When 10 mg/L (final concentration) CFW was added to the culture, a significant down shift of the growth rate was found (red growth curve in Fig. 2). Although the effect on growth inhibition increased with higher concentrations of CFW in the culture media, this effect was not linear (data not shown). Even at 100 mg/L mass concentration of CFW, complete inhibition of growth could not be achieved (blue growth curve in Fig. 2). This result is consistent with previous findings[14]. This result also indicates that simply increasing the dose of CFW is not a viable option to improve the drug's performance. De Nobel et al.[15] showed that when wild-type yeast grew in the presence of CFW (10 mg/L), a progressive increase in the level of phospho-Slt2 was detected. Slt2 is the key transcription factor in yeast for CFW interference, so we selected 10 mg/L CFW for subsequent analysis.

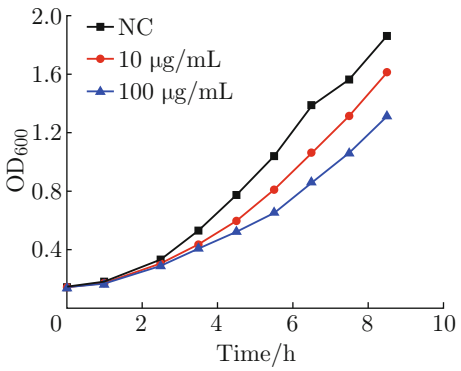
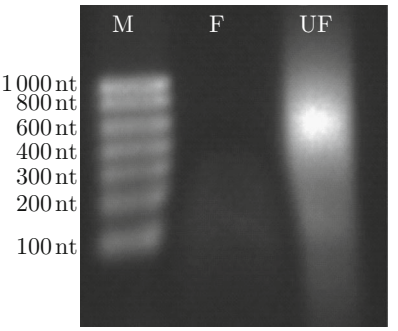


Fig. 2 Growth rate of *saccharomyces cerevisiae* under CFW treatment

2.2 Generating CFW Induced *Saccharomyces Cerevisiae* mRNA Profiles Using Affymetrics GeneChip Yeast Genome 2.0 Microarray

Extracted total RNA was prepared for hybridization according to the user's manual of the Affymetrix GeneChip 3'IVT express kit. The length distribution of the prepared aRNA was checked with gel shift assays. As shown in Fig. 3, before aRNA fragmentation, aRNA profile was a distribution with most of the aRNA between 500—1 000 nucleotide (nt). After aRNA fragmentation, the distribution was shifted to a much shorter and consistent with the peak at approximately 150 nt.



M—RNA marker, F—Fragmented aRNA
UF—Un-fragmented aRNA

Fig. 3 The agarose gel to show the size distribution of aRNA before and after fragmentation

On the Affymetrics GeneChip Yeast Genome 2.0 microarray, 5 845 protein coding genes of *saccharomyces cerevisiae* are present, which are selected from the public data source GenBank® (May 2004). With our samples, signals have been found on all of these genes after the initial processing, using the RMA algorithm for background correction, normalization and summarization. The scatterplot between two biological replicates of 10 mg/L CFW treated culture is shown in Fig. 1.

When comparing with the control, using the 1.5 fold change as the cutoff and the P-value below 0.05, we identified 99 genes that were up-regulated and 7 genes down regulated after *saccharomyces cerevisiae* was treated with 10 mg/L CFW for 3 h (gene up-regulated, Table 1). The gene ontology of these differentially expressed genes was also summarized in

Table 1 ***Saccharomyces cerevisiae*** **genes differentially expressed upon CFW treatment**

Fold change	Annotated function	Gene name
< -1.5	Stress response genes (14%)	WSC4
	Other functions (43%)	LAP4, ANB1, HEM13
	Unknown function (43%)	ICS2, 2 genes
> 1.5	Cell wall related genes (17%)	PIR3, CWP1, HPF1, KTR2, MCH5, GND2, PST1, OPT2, ECM4, HBT1, CRH1, DFG5, PLB1, HMX1, CHS1, CHS3, HSP12
	Cell wall integrity pathway (16%)	BAG7, PRM5, YPK2, YPS3, YPS6, SLT2, PTP2, MTL1, FLC2, GPH1, SSK22, GSC2, HPF1, PRM5, PRM10, AFR1
	Stress response genes (13%)	HSP1, DDR2, SIP18, YKE4, UGX2, ALD3, FMP16, NCA3, MBR1, XBP1, SPG4, USV1, DBF2
	Metabolism (10%)	AMS1, FBP26, GCY1, RNR3, NQM1, NDE2, GLC3, GAC1, GIP2, SGA1
	Other functions (13%)	CRG1, SRL3, GPG1, PUT4, SOM1, CAR2, HUG1, PAU2, FMP46, HPA2, SOR1, AGX1, ADR1
	Unknown function (31%)	FMP33, DDI2, MOH1, PET10, 26 unknown genes

Table 1. About 33% of these genes were related to cell wall synthesis or related functions, 13% were related to stress response functions, 10% were related to metabolism of the cell, and 44% of these genes were either of unknown function or could not be clearly assigned.

2.3 Biological Process and Pathway Enrichment Analysis of Up-Regulated Genes Induced by CFW

We conducted gene ontology analysis (DAVID Tool) to identify significantly enriched GO functional terms for the 99 up-regulated genes induced by 10 mg/L CFW for 3 h. These results were presented in Tables 2 and 3. Our data indicate that under the "biological process" term (category goterm_bp_fat, Table 2), highly enriched GO groups were mainly related cell wall integrity, glycogen and saccharide metabolic processes. Under the cell component category, the up-regulated *saccharomyces cerevisiae* genes induced by CFW were also related to cell membrane and cell wall integrity (category goterm_cc_fat, Table 3).

These analyses indicate that although the cell wall related genes are most enriched in the up-regulated transcripts as expected owing to the direct effect of CFW, additional pathways are indeed also involved when the cell is attempting to compensate the stress induced by CFW. The same conclusion is also reached by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (Table 4).

Table 2 Enriched GO terms under the biological process category for up-regulated *saccharomyces cerevisiae* genes induced by 10 mg/L CFW

Term	Count	P-value	Benjamini (i.e., adjusted P-value)
GO:0009628,response to abiotic stimulus	29	4.49×10^{-19}	2.01×10^{-16}
GO:0009266,response to temperature stimulus	24	8.83×10^{-19}	1.98×10^{-16}
GO:0009408,response to heat	23	3.91×10^{-18}	5.84×10^{-16}
GO:0034605,cellular response to heat	22	8.52×10^{-18}	9.54×10^{-16}
GO:0007047,cell wall organization	18	7.06×10^{-10}	6.32×10^{-8}
GO:0045229,external encapsulating structure organization	18	7.40×10^{-10}	5.52×10^{-8}
GO:0005996,monosaccharide metabolic process	11	3.93×10^{-6}	2.51×10^{-4}
GO:0005976,polysaccharide metabolic process	9	8.27×10^{-6}	4.63×10^{-4}
GO:0019318,hexose metabolic process	10	9.62×10^{-6}	4.79×10^{-4}
GO:0033554,cellular response to stress	25	2.85×10^{-5}	12.74×10^{-4}
GO:0019953,sexual reproduction	14	3.15×10^{-5}	12.83×10^{-4}
GO:0007039,vacuolar protein catabolic process	8	6.37×10^{-5}	23.75×10^{-4}
GO:0005977,glycogen metabolic process	5	9.87×10^{-5}	33.95×10^{-4}
GO:0006006,glucose metabolic process	8	1.15×10^{-4}	36.67×10^{-4}
GO:0006073,cellular glucan metabolic process	6	1.60×10^{-4}	47.65×10^{-4}
GO:0044042,glucan metabolic process	6	1.60×10^{-4}	47.65×10^{-4}
GO:0044264,cellular polysaccharide metabolic process	7	2.90×10^{-4}	80.87×10^{-4}
GO:0006112,energy reserve metabolic process	5	3.74×10^{-4}	98.11×10^{-4}
GO:0048610,reproductive cellular process	11	13.09×10^{-4}	32.07×10^{-3}
GO:0016052,carbohydrate catabolic process	7	15.75×10^{-4}	36.48×10^{-3}

Table 3 Enriched GO terms under the cell component category for up-regulated *saccharomyces cerevisiae* genes induced by 10 mg/L CFW

Term	Count	P-value	Benjamini (i.e., adjusted P-value)
GO:0031225,anchored to membrane	10	1.87×10^{-8}	1.78×10^{-6}
GO:0005618,cell wall	10	1.45×10^{-6}	6.91×10^{-5}
GO:0030312,external encapsulating structure	10	1.45×10^{-6}	6.91×10^{-5}
GO:0031224,intrinsic to membrane	34	2.12×10^{-6}	6.71×10^{-5}
GO:0009277,fungal-type cell wall	9	3.33×10^{-6}	7.92×10^{-5}
GO:0000267,cell fraction	11	5.80×10^{-6}	1.10×10^{-4}
GO:0005624,membrane fraction	9	8.95×10^{-5}	14.17×10^{-4}
GO:0005626,insoluble fraction	9	8.95×10^{-5}	14.17×10^{-4}
GO:0005886,plasma membrane	15	2.32×10^{-4}	31.38×10^{-4}
GO:0005576,extracellular region	7	3.98×10^{-4}	47.16×10^{-4}
GO:0046658,anchored to plasma membrane	3	17.04×10^{-4}	17.84×10^{-3}
GO:0031226,intrinsic to plasma membrane	5	21.58×10^{-4}	20.31×10^{-3}
GO:0001950,plasma membrane enriched fraction	5	29.53×10^{-4}	25.22×10^{-3}

Table 4 KEGG pathways for up regulated *saccharomyces cerevisiae* genes induced by 10 mg/L CFW

Term	Count	*P*-value	Benjamini (i.e., adjusted *P*-value)
sce00500: starch and sucrose metabolism	4	18.80×10^{-4}	33.30×10^{-3}
sce00051: fructose and mannose metabolism	3	12.41×10^{-3}	10.63×10^{-2}
sce04011: MAPK signaling pathway	3	54.33×10^{-3}	28.48×10^{-2}

3 Discussion

CFW is a broad spectrum antifungal compound with very low toxicity for humans. However, its clinical application in treating fungal infection is hampered by its limited efficacy, owing to fungal cells' effective compensatory responses. A full understanding of its compensation mechanism is the basis to further develop this drug into an effective treatment approach, used either alone or in combination with other drugs[8]. In this study, we have used microarray analysis to dissect the responses of *saccharomyces cerevisiae* when treated by CFW to identify cellular pathways important for cell survival.

Using a stringent criterion, we have found more than 100 protein coding genes that are differentially expressed upon CFW treatment, with only 7 that are down regulated. The up-regulated genes are mostly cell wall related genes and genes for energy metabolism. This is consistent with the notion that CFW perturbs the cell wall integrity and causes energy utility imbalance (Table 1). Since the 7 down regulated genes are either of unknown function or inessential for *saccharomyces cerevisiae*, we mainly focus on the up-regulated genes in the following discussion.

CWF is a chitin antagonist and binds chitin to compromise the assembly of the cell wall. Among the cell wall related genes, we found that chitin synthesis related genes, including chitin synthase I (CHS1) and chitin synthase III (CHS3), are up-regulated after CWF treatment. CHS1 and CHS3 are chitin synthase genes, which catalyze the transfer of N-acetylglucosamine (GlcNAc) to chitin. Additionally, chitin transglycosylase CRH1 is also up-regulated, which can transfer chitin to 1,6-beta and 1,3-beta glucans in the cell wall. Besides chitin, other *saccharomyces cerevisiae* cell wall components and related genes are also up-regulated. The transcript abundance of the cell wall mannoprotein (CWP1) is increasing upon CFW treatment. CWP1 is localized to birth scars of daughter cells and links to a 1,3-beta and 1,6-beta glucan heteropolymer through a phosphodiester bond. Another up regulated gene GSC2 (i.e., 1,3-beta-glucan synthase) is involved in the formation of the inner layer of the spore wall. These results clearly indicate that in addition to the direct target of CFW, other cell wall components must also be up-regulated by the cell to compensate the damage.

Our data also show that the key components of the PKC-SLT2 pathway are up-regulated upon CWF treatment as well. It has been known that PKC-SLT2 pathway is one of the important pathways for cell wall integrity. Since BAG7 (i.e., Rho GTPase activating protein) is up-regulated with CFW binding to chitin, this protein can activate Rho1p by stimulating its intrinsic GTPase activity. In turn, Rho1p activation leads to the activation of the mitogen-activated protein kinase (MAPK) cascade through Pho1p activation of Pkc1p. The MAPK cascade then activates serine/threonine MAP kinase SLT2. In addition to this possible activation of SLT2, we also found that the transcript of SLT2 is up-regulated. Although this protein requires an upstream kinase to be activated, an increase in the protein amount provides a sufficient pool of targets for the upstream kinase. It is known that activated SLT2 can rapidly, but transiently accumulate in the nuclear compartment to active transcription factor RLM1 (i.e., MADS-box transcription factor). And RLM1 is thought to participate in a modification of the transcriptional pattern in response to cell-wall integrity stress.

In addition, changes in the high osmolarity growth (HOG) pathway, such as the MAPKKK (i.e., mitogen-activated protein kinase kinase kinase) of gene SSK22 (i.e., HOG1 mitogen-activated signaling pathway) is also interesting. Normally, one would assume that HOG pathway activation would be a specific response to osmolarity abnormalities. The up regulation of SSK22 upon CFW incubation would suggest that the changes in the cell wall could also impose certain stress on the osmotic regulators which leads to the increased expression of SSK22, although the details of the molecular pathway can remain to be elucidated.

Furthermore, our data have indicated that the cell's response to cell wall stress is not restricted to those pathways or processes related to the cell wall or the plasma membrane. In fact, genes in the glycogen and hexose metabolism pathway are also up-regulated upon CFW treatment. Several glycogen synthesis process related genes are up-regulated after CWF treatment. For example, serine/threonine-protein phosphatase 1 regulatory subunit (GAC1) is up-regulated, which regulates the activity of glycogen synthase. And 1,4-alpha-glucan-branching enzyme (GLC3) is also up regulated. This enzyme is responsible to the transfer of a segment of a (1-> 4)-alpha-D-glucan chain to a primary hydroxy

group in a similar glucan chain. As glucose is a major carbon and energy source for the fungi, one might speculate that cell wall compromise could require a heightened metabolism to effectively deal with the cell wall repair and other related stress.

4 Conclusion

Our results have firmly established that the effect of CFW is not confined to those biological processes directly involved in the synthesis of chitin, which is the direct target of CFW. It is reasonable to hypothesize that these additionally up-regulated genes are also of critical importance for the survival of the fungi. Therefore, targeting these pathways or components may provide the possibility to substantially improve the efficacy of CFW when applied in conjunction. Even if some of the targets may be present in the host cells, a sub-lethal level of application of these additional compounds may be sufficient to substantiate the effect of CFW, leading to more effective regimens of treatment for fungal infections in human patients.

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