Curcumin targets cell wall integrity via calcineurin mediated signaling in *Candida albicans*.

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Running Title: Antifungal mechanism of action of curcumin.

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ABSTRACT

Curcumin (CUR) shows antifungal activity against a range of pathogenic fungi including *Candida albicans*. The reported mechanisms of action of CUR include ROS generation, defects in ergosterol biosynthesis pathway, decrease in hyphae development and modulation of multidrug efflux pumps. Reportedly, each of these pathways is independently linked with the cell wall machinery in *C. albicans* but surprisingly, CUR is not previously implied in connection with cell wall damage. In the present study, we have performed a transcriptional profiling to identify the yet unidentified targets of CUR in *C. albicans*. We found that, among 348 CUR affected genes, 51 genes were up regulated and 297 genes were down regulated. Interestingly, most of the cell wall integrity pathway genes were down regulated. The possibility of cell wall playing a critical role in the mechanism of CUR required further validation; therefore we performed specific experiments to establish any link between the two. The fractional inhibitory concentration index values of 0.24 - 0.37 show that CUR interacts synergistically with cell wall perturbing (CWP) agents (Caspofungin, Calcofluor White, Congo Red and SDS). Furthermore, we could observe cell wall damage and membrane permeabilization by CUR alone as well as synergistically with CWP agents. We also found hypersusceptibility in calcineurin and MAP kinase pathway mutants against CUR, which confirmed that CUR also targets cell wall biosynthesis in *C. albicans*. Together, these data provide strong evidences which show that CUR disrupts cell wall integrity in *C. albicans*. This new information of mechanistic action of CUR could be employed in improving treatment strategies and in combinatorial drug therapy.
INTRODUCTION

Candidiasis caused due to Candida albicans is the most common opportunist fungal infection and a serious medical problem that causes significant morbidity and mortality, particularly in AIDS patients, transplant recipients and other immunocompromised people (1). In spite of the continuous expansion of the arsenal of antifungal drugs, the available drugs cannot meet the ever increasing requirements for combating Candida infections in the patients. Infections caused by pathogenic C. albicans are commonly treated either by azoles or some non-azole antifungal agents but they have more pronounced side effects (2, 3). In clinical setup, excessive usage of these antifungals causes liver-damage, altered estrogen levels, allergic reactions and above all development of drug resistance among patients (4). Hence, there has been an urgent need to develop effective drugs for combating against these fungal diseases.

Researchers in the recent years have turned their attention towards utilizing natural products as the building blocks for the development of the effective next generation drugs. Curcumin (CUR; Mixture of curcuminoids) is extracted from rhizome of Curcuma longa (a small perennial herb native to India) and known to have therapeutic potential due to its anti-inflammatory, anti carcinogenic, and anti-infectious properties (5, 6). It has long been used as a common household medicine and as a spice in Southeast Asia and earlier, we had reported its antifungal activity against C. albicans (7). An antifungal activity of CUR is also reported against Cryptococcus neoformans, Sporothrixschenckii, Paracoccidioidesbrasiliensis and Aspergillus spp. (5). Reports show that antifungal activity of CUR occurs via multiple pathways in C. albicans. For example, CUR leads to cell death via Reactive Oxygen Species (ROS) induced apoptosis in C. albicans (8). This ROS induced cell killing was also observed when CUR was used synergistically along with azoles and polyenes (7). In another study, CUR was shown to
target ERG3 of *C. albicans* leading to altered ergosterol biosynthesis, which in turn is critical to drug resistance (9). Additionally, the activity of multidrug efflux pumps namely CaCdr1p, CaCdr2p, CaMdr1p and ScPdr5p, have been shown to be modulated by CUR (10). Overall, CUR targets majority of the pathways known to be directly involved in acquisition of drug resistance.

Studies show that there is a linkage between the cell wall and induction of drug resistance in *C. albicans*. There is strong evidence that protein kinase C (PKC) signaling, which comprises of Hsp90 and calcineurin, governs drug resistance phenomenon of *C. albicans* (11). Also, resistance to cell wall targeting drugs like echinocandins (a non-azole drug) have been shown to develop through calcineurin mediated signaling (3) and through 1, 3-glucan biosynthesis inhibition (12) in *C. albicans*.

Considering the above mentioned facts, one may question whether CUR has any link with cell wall pathways of *C. albicans*. In the present study we have used microarray approach to identify global changes that occur upon CUR treatment in *C. albicans*. Interestingly, apart from the other known target pathways of CUR, many genes related to cell wall biogenesis were also found to be downregulated. This result was in line with our earlier presumption that CUR may be targeting cell wall of *C. albicans*. Therefore, we have extended this study to discuss the mode of action of CUR related to cell wall disruption. Interestingly, CUR alone and in combination with cell wall perturbing (CWP) agents (like Caspofungin (CAS), Calcofluor White (CFW), Congo Red (CR) and SDS) at its synergistic concentration modulates cell wall architecture of *C. albicans*. Using mutant variants of calcineurin and MAP Kinase pathway, we were able to show that CUR targets cell wall integrity and promotes cell death by disrupting the cell wall of *C. albicans*. 
MATERIALS AND METHODS

Chemicals used. Most media components were obtained from HiMedia (Mumbai, India) while bacto agar was obtained from Difco (BD Biosciences, NJ, USA) and RPMI-1640 media from Gibco BRL (Gaithersburg, MD, USA). Calcofluor white (CFW; 4-methyl-7-diethylamino Coumarin), Curcumin (CUR: commercial grade mixture of curcuminoids), Congored (CR), Sodium dodecylsulphate (SDS), Sodium Chloride and other chemicals used in the experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Propidium iodide (PI) was purchased from Molecular Probes (Eugene, USA).

Candida strains, media and growth condition. All C. albicans strains/mutants used in this study are listed in supplementary table S1. All strains were maintained on the Yeast-Extract-Peptone-Dextrose (YEPD) broth and agar plate (HiMedia, Mumbai, India) at 30°C.

Cell culture and CUR exposure for microarray experiment. Candida cells were grown at 30°C in YEPD medium with to an optical density at 600 nm (OD600) of 0.1 and were then split into two cultures. For CUR treatment 0.1 OD cells were allowed to reach 0.4 OD and then 251 µM (92.45 µg/ml) of CUR (MIC50) was added to one of the cultures, and the second culture was subjected to an equivalent mock treatment (water). After 14h CUR treatment, the cells were harvested and used for RNA isolation (13). Three independent cultures were prepared for biological repeats.

RNA isolation, cDNA synthesis, labelling and hybridization. RNA isolation was done by following Trizol (Sigma) method as per manufacturer’s specification except that 300 µl acids washed 0.4-0.6 mm glass beads (Sigma, St. Louis, MO, USA) were used during cell lyses (14). 10 mg of purified total RNA was used to synthesize cDNA by using the protocol described in www.transcriptome.ens.fr/sgdb/protocols/labelling.yeast.php. We used previously described
direct labeling method to label cDNA with Cy3 and Cy5 dyes (15). Labeled cDNA was mixed with hybridization buffer and applied immediately on the microarray slides. It was covered 22*60 mm cover slips, sealed in Corning hybridization chambers and allowed to incubate overnight at 42 °C. Slides were washed and scanned. Each experimental condition was independently repeated thrice including a dye swap. *C. albicans* microarrays (batch C050G) were obtained from Eurogentec (Seraing, Belgium) containing cDNA probe deposited in duplicate, for 98% of *Candida* open regarding frames (assembly 19) (13).

**Scanning and cDNA microarray data analysis.** Slides were scanned using Scan Array Express microarray scanner by Perkin Elmer. Raw data obtained was quantified using adaptive circle method and normalized using LOWESS. The value obtained by this was background corrected and ratio of treated versus untreated intensity were converted in Log$_2$ ratios which was averaged for replicates of same set of experiment. We have used www.candida.bri.nrc website to obtain the annotations for the ORFs. To address the biological description and relevance of the genes presented in table S2 of the supplemental material, we looked for gene functional categories in the *Candida* Genome Database (CGD) and gene ontology term finder tools (16).

**Reverse transcriptase PCR (RT-PCR).** For validation of the microarray results, RT-PCR was done as mentioned in the RevertAid™ H Minus kit (MBI, Fermentas). The amplified products were subjected to gel electrophoresis and were quantitated by using Quantity One software with the Bio-Rad gel documentation system. The densities of bands (for genes of interest) were measured and normalized to that of the endogenous gene Actin (*ACT1*). We have taken biological replicated and standard deviation of the intensity per unit area to validate RT-PCR. Primers used in this experiment are listed in supplementary table S3.
Determination of minimum inhibitory concentration (MIC). MICs of CWP agents (CAS, CFW, CR and SDS) against the *C. albicans* strain SC5314 was determined by broth microdilution using two-fold serial dilutions in RPMI-1640 medium, as described in method M27-A3 by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS, USA) (17).

**Chequerboard assay.** The interaction of CUR with CWP agents was evaluated by the chequerboard method recommended by the CLSI (7) and expressed as the sum of the fractional inhibitory concentration index (FICI) for each agent. Chequerboard assay was performed in triplicate as described earlier (8). The FICI of each agent was calculated as the MIC of such agent in combination divided by the MIC of such agent alone (18). The FICI was interpreted as synergistic when it was $\leq 0.5$, antagonistic when it was $\geq 4.0$, and any value in between was interpreted as indifferent (12).

**Time kill assay.** *C. albicans* cells (~$10^6$) were inoculated in RPMI-1640 medium containing CUR, CFW, CR, and SDS alone and in combination. The concentrations of drugs used here are the synergistic concentration. At pre-determined time points (0, 1, 4, 8, 12, 16, 20, 24 h at $30^\circ$C incubation; agitation 200 rpm), a 100 µl aliquot was removed, serially diluted (10 fold) in saline and plated on YPD agar plates. Colony counts were determined after incubation at $30^\circ$C for 24 h in triplicate (19).

**Growth percentage assessment.** The interaction of CUR with different CWP agents was evaluated by the growth curve via three independent experiments. After inoculation $10^4$ cells in RPMI-1640 medium and synergistic concentration of mentioned drugs (alone and in combination with CUR) in the microdilution plates, it was agitated for 15 s and incubated at $37^\circ$C for 24 h and OD$_{600\text{nm}}$ was recorded. The changes in OD over time were used to generate growth percentage at each drug concentration and for the drug-free growth control.
Spot assay. Drug susceptibilities of CUR, CFW, CR, and SDS against SC5314 strain of *C. albicans* in solid medium were measured using spot assay method as described previously and differences were monitored after growth for 48 h at 30°C (20). Spot assay was also performed with Calcineurin, MAP kinase and HSP90 pathways mutants (Supplementary table S1).

Confocal microscopy. *Candida* cells (~10⁶) were suspended in RPMI 1640 medium and incubated with Propidium Iodide (PI) 1.5µM + CUR alone at their MIC₈₀ and PI+CUR+ CWP agents at synergy concentration on 30°C with constant shaking (200 rpm) for 4 h visualized by confocal microscopy (Olympus Fluoview™ FV1000) at wavelength >560 nm (20). MIC₈₀ of CUR was used but not MIC₅₀, as in transcriptome profiling experiment to visualize the dye uptake.

Electron microscopy. Cell damage was observed in CUR treated cells (~10⁶) at MIC₁₀₀ concentrations and CUR + CWP agents at synergistic concentrations using scanning and transmission electron microscopy [SEM (Zeiss EV040) and TEM (JEOL, 2100F), respectively]. MIC₁₀₀ of CUR was used to compare if combination of CUR and CWP at synergistic concentrations can bring about the same impact on the cell wall. Sample preparation and analysis methods were similar to as described previously (20). The images were acquired with 30000 X magnifications.

RESULTS

Transcriptional response of the *C. albicans* to CUR. To obtain a detailed overview of mechanistic action of CUR, transcriptome profiling of *C. albicans* was performed at MIC₅₀ (251 µM; 92.45 µg/ml) concentration of CUR only. We used MIC₅₀ for the array experiments to avoid the growth defects/inhibition that would have occurred at higher MIC values and we found that MIC₅₀ concentration was just sufficient to trigger the transcriptional response. Microarray data
analysis showed that 348 genes were differentially expressed in CUR-treated *C. albicans* cells (SC5314) as compared with *C. albicans* wildtype strain untreated with CUR. 51 genes were significantly upregulated and 297 genes were significantly downregulated among them. Cut-off value in terms of log2 ratio was mentioned in table S2 of the supplemental material.

Transcriptome data showed that CUR on *C. albicans* was effective via multiple pathways including cell cycle, cell signaling, cell wall integrity, cellular metabolic processes, stress, cytoskeletal organization, DNA synthesis/repair, hyphal development, mitochondria, transcription, translation, transport, virulence and several uncharacterized genes (Fig. 1). Further, to validate the microarray data, we performed RT-PCR in response to CUR under similar growth conditions. We randomly picked up few genes, both up- and down-regulated, for RT-PCR analysis and found that the RT-PCR data corroborates with the microarray results (Supplementary Fig. S1).

**CUR targets the genes cell wall integrity pathway.** Among the categories of genes that were found to be downregulated upon CUR treatment, we found prominent changes in the genes that maintain cell wall integrity in *C. albicans* (Supplementary Table S2). Although cell wall integrity pathway is known to play a significant role in combating various drug treatment (21), but surprisingly no study till date has shown any link between CUR and cell wall. We found that upon CUR exposure, eleven genes of this class were downregulated. Specifically, *PGA28, ECM39, GFA1, HWP2, PMT4, HYR3, HDA1, CHSI, CHT1, orf19.5271 and orf19.376* were significantly downregulated upon CUR treatment (Fig. 1). This result suggests that the cell wall integrity might be compromised in *C. albicans* when exposed to CUR. We have also analyzed the enrichment for GO terms, where cell wall category was found to be enriched (Supplementary...
Therefore, in the later part of this study we specifically focused to discuss the link between CUR and cell wall integrity.

**Antifungal activity of the CUR and CWP agents.** To elaborate the microarray results that suggest implications of CUR on the cell wall stress, we first determined the antifungal activity (in terms of MIC$^{100}$) of CUR and CWP agents against *C. albicans*. The MIC was defined as the lowest concentration of drug exhibiting 100% inhibition of growth compared with that of the drug-free control. The MIC$^{100}$ for CUR was 695 µM (256 µg/ml), CAS was 0.39 µM (0.42 µg/ml), CFW was 62.5 µM (57.5 µg/ml), CR was 15.7 µM (11 µg/ml) and SDS was 250 µM (72 µg/ml).

**CUR is synergistic with CWP agents.** *In vitro* antifungal effects of CUR in combination with CWP agents (CFW, CR and SDS) were tested against *C. albicans*. The results showed that combinations of non-candidacidal concentrations of CUR and CWP agents were highly active against *C. albicans*, suggesting their synergistic action. Combination of non-candidacidal concentrations of 86.8 µM (31.97 µg/ml) of CUR + 0.048 µM (0.051 µg/ml) of CAS, 173.7 µM (63.98 µg/ml) CUR + 1.95 µM (1.79 µg/ml) of CFW, 173.7 µM (63.98 µg/ml) CUR + 1.90 µM (1.36 µg/ml) CR and 86.8 µM (31.97 µg/ml) CUR + 31.25 µM (9 µg/ml) SDS showed almost complete killing of *C. albicans*. FICI values of CUR with CWP agents were determined as described in methods. As shown from the FICI values, CUR was synergistic with all tested CWP agents (Table 1). In combination with CUR, MIC$^{100}$ of CWP agents was reduced to 8 fold for CAS, 32 fold for CFW, 8 fold for CR and SDS and MIC$^{100}$ of CUR was reduced from 4-8 fold with CWP agents.

**Time-kill curves, growth percentage assessment and spot assay reconfirm synergism of CUR with CWP agents.** For validation of synergy result, we performed time kill assay,
growth percentage assessment and spot assay that further confirmed the synergistic interaction of CUR and CWP agents (Fig. 2). Time kill curves showed that CUR and either of the tested CWP agents at the indicated concentrations alone did not affect the growth of *C. albicans* (Fig. 2A). However, a combination of CUR with CWP agents significantly affected the CFU count (Fig. 2A) as well as the percentage cell growth of *C. albicans* after 24 h (Fig. 2B). Solid medium spot assay also showed synergy of CUR with CWP agents in *C. albicans* (Fig. 2C). The data from these experiments confirmed the high susceptibility of the *C. albicans* tested with CUR in combination of CWP agents, indicating synergistic action.

**CUR induced membrane permeabilization in *C. albicans***. Alteration in membrane permeability indicates a change in physical state of the membrane or towards compromised cell wall integrity. To determine whether CUR treatment induces membrane permeabilization in *C. albicans*, we have measured the uptake of membrane impermeant dye, PI. Incubation of CUR (MIC$_{80}$) with *Candida* cells resulted in PI uptake as monitored by confocal microscopy (Fig. 3). We also observed PI uptake in *C. albicans* cells when CUR was used in combination with CWP agents at synergistic concentration (Fig. 3). This result showed that when used in combination, CUR acted synergistically with CWP agent to cause membrane permeabilization at concentrations that are very low compared to their individual MIC values. No PI uptake was observed when *C. albicans* cells were treated with PI alone or with a non-antifungal concentration (MIC$_{50}$) of CUR or CWP agents (control).

**CUR treatment causes abrogation of cell surface morphology in *C. albicans**. Higher membrane permeability could be due to altered cell surface morphology of *C. albicans*. To verify this fact, we looked for morphological changes in CUR treated *C. albicans* cells by SEM (Fig. 4). Cells treated with CUR alone, at their respective MIC$_{80}$ values for 24 h, showed wrinkling
and corrugation of the cell surface, compared with the smooth surface of untreated cells (Fig. 4A). Further, we assessed morphological changes in *C. albicans* cells when treated with CUR and CWP agents in combination (CUR+CFW, CUR+CR, CUR+SDS) at non-candidacidal synergistic concentrations (Fig. 4 B, C, D). We found that upon treatment of CUR + CWP agents at synergistic concentration, cells showed wrinkling and corrugation (shown by arrow) of the cell surface (Fig. 4Biv, Civ, Div). In the control experiment, the untreated cells (Fig. 4 Bi, Ci, Di) or treated alone with synergistic non-fungicidal concentration of CUR (Fig. 4 Bi, Ci, Di), CFW (Fig. 4 Bii), CR (Fig. 4 Cii), SDS (Fig. 4 Dii), showed a smooth cell surface. These data showed that CUR in combination with CWP agents at synergistic concentrations could cause extensive damage to the cell surface morphology.

**CUR mediated cell wall damage in *C. albicans***. To get a detailed insight into the structural changes induced upon CUR treatment, we monitored the ultra-structure of *C. albicans* cells using TEM (Supplementary Fig. S2). Ultrathin section of CUR treated (MIC100) *C. albicans* cells showed damage and alteration in the cell wall (Supplementary Fig. S2 A). We also found alteration and breakage in cell wall of *Candida* cells after treatment of CUR in combination with CWP agents at synergistic concentrations (Supplementary Fig. S2 Biv, Civ, Div). At non-candidacidal concentrations of CUR and CWP agents the cell surface is intact just like untreated cells (Supplementary Fig. 2 Bi, Ci, Di). These results strengthen the fact that CUR might also be targeting cell wall to kill *C. albicans* cells.

**CUR confers hyper-susceptibility in the mutants of calcineurin and MAP kinase pathway in *C. albicans***. Our microarray results and drug susceptibility assays suggested that CUR might be targeting critical genes of the cell wall integrity pathways. Reportedly, calcineurin and MAP Kinase are two well established pathways required for the maintenance of cell wall
integrity in *C. albicans* and several other fungi (22, 23, 24). Therefore, the mutants of *C. albicans* calcineurin signaling pathway ($\Delta$emp1/$\Delta$emp1, $\Delta$cnb1/CNB1, $\Delta$cnb1/$\Delta$cnb1, $\Delta$crz1/$\Delta$crz1), MAP kinase pathway ($\Delta$bck1/$\Delta$bck1, $\Delta$mkc1/$\Delta$mkc1) and stress pathway ($\Delta$hsp90A/HSP90) were examined for the effect of CUR (2.37 mM, non-fungicidal concentration) treatment. All the tested mutants of calcineurin and MAP kinase mutants exhibited hyper-susceptibility to CUR (Fig. 5). Moreover, the observed effect of CUR was comparable to the growth of these mutants in the presence of CFW (Fig. 5), a well known agent that compromises cell wall integrity by binding to chitin (25). The data suggests that CUR disrupts cell wall integrity by targeting MAP kinase pathway and calcineurin mediated signaling.

**DISCUSSION**

Previously we demonstrated the antifungal activity of CUR against *C. albicans* and postulated that several mechanisms are responsible for the cell death (7-10). Many of these mechanisms are also crucial for the development of drug tolerance in *C. albicans* (26). In continuation towards understanding the possible mechanism of action of CUR at a molecular level, in the present study we performed a microarray study in *C. albicans* in response to CUR treatment. Our microarray analyses further showed that CUR employs multiple mechanisms to kill the cells viz cell cycle, signaling alteration, loss of cell wall integrity, metabolic shift, cell stress, DNA synthesis and repair, hyphal development, mitochondrial integrity, transcriptional and translational regulation etc. (Supplementary table S2, Fig. 1). Among these multiple mechanisms, CUR shows a significant influence on the genes belonging to cell wall integrity because most because most of the genes of this pathway were downregulated.
By taking the lead from microarray results, we extended and verified CUR mediated cell wall damage hypothesis through other experiments (described in materials and methods). We firstly tested the synergistic effect of CUR with some known cell wall perturbing agents (CFW, CR and SDS) CUR synergized the effect of CWP agents which suggests that the cell wall is a major target of CUR in cell death mechanism of *C. albicans*. We also found the loss of cell viability at synergistic concentrations (of CUR and CWP agents) by the time kill, growth and spot assays; showing that both CUR and CWP agents share a common target. Overall, these results validate the microarray outcome and confirm that CUR is involved in *C. albicans* cell wall damage.

Alteration of membrane permeability is one of the major mechanisms underlying the drug tolerance phenomenon and damage of cell wall should affect the overall cell permeability (27). We found that cell membrane become permeable to a membrane impermeant dye, PI (28), when cells were exposed to CUR alone with CWP agents at synergistic concentration. We could further find extensively altered cell surface morphology and cell wall ultra structure of *C. albicans* after CUR treated in SEM and TEM study to gain further insights into the cell wall perturbation mechanisms of CUR. Fungal growth, development and the ability to survive in environmental stress conditions are dependent on the integrity of the cell wall (26). Reports show that calcineurin plays an important role in governing the cell wall integrity which might involve related pathways such as the PKC, HSP90 (2, 29, 30, 31). Earlier, LaFayette *et al.*, (2010) described the level of regulatory control circuitry that links PKC and calcineurin signaling and governs the cell wall integrity pathway in the *C. albicans* (11). Our data showed that calcineurin and MAP kinase pathway mutants of *C. albicans* are hypersusceptible to CUR, suggesting that these genes render fungicidal activity of CUR in and play a critical role in the mechanism of
CUR induced cell wall integrity defect of *C. albicans*. Phenotypic susceptibility assays in response to CUR in calcineurin mutants validated the microarray data. Evidently, both cell wall integrity pathway and calcineurin pathway are working against CUR stress but whether these pathways are connected or work independently towards CUR stress remains to be validated. Together, our data displays that CUR in *C. albicans* causes direct damage to the cell wall and loosening of membrane permeability which finally leads to cell death by leakage of cytoplasmic content of the cells. Fig. 6 summarizes the known mechanism of CUR mediated cell death along with our recent findings that affect cell wall integrity in *C. albicans*.

Although the upcoming cell wall targeting drugs have shown great potential in treating *Candida* infections but many cases of development of resistance to these drugs are already coming up (7). Combinational drug therapy is an alternate and effective strategy to treat Candidiasis at much lower concentration of drugs compared to what is used individually. In the present study, we were able to show that CUR synergizes the affect of CWP agents by targeting the cell wall in *C. albicans*. Thus the doses of drugs like candins if given in combination with CUR could be significantly reduced in clinical settings. Further this study highlights some important mechanisms of anti-*Candida* effects of CUR which include loss of cell viability, membrane permeability and ultra-structural alteration of cell wall. Together, our data provided the mechanistic insights that demonstrate a link between CUR susceptibility of *C. albicans* with calcineurin signaling pathway and presenting an opportunity to employ this new information in improving the treatment strategies.

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REFERENCES


Table 1: FICI values of CWP agents with CUR against C. albicans.

<table>
<thead>
<tr>
<th>CUR alone (µg/ml)</th>
<th>CWP agent alone (µg/ml)</th>
<th>Conc. of CUR (µg/ml) and CWP agent (µg/ml) in combination</th>
<th>FICI</th>
<th>Effect</th>
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<tbody>
<tr>
<td>256</td>
<td>0.42 (CAS)</td>
<td>31.97 (CUR) + 0.051 (CAS)</td>
<td>0.24</td>
<td>Synergy</td>
</tr>
<tr>
<td>256</td>
<td>57.5 (CFW)</td>
<td>63.98 (CUR) + 1.79 (CFW)</td>
<td>0.28</td>
<td>Synergy</td>
</tr>
<tr>
<td>256</td>
<td>21.85 (CR)</td>
<td>63.98 (CUR) + 1.36 (CR)</td>
<td>0.37</td>
<td>Synergy</td>
</tr>
<tr>
<td>256</td>
<td>72 (SDS)</td>
<td>31.97 (CUR) + 9 (SDS)</td>
<td>0.25</td>
<td>Synergy</td>
</tr>
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LEGENDS OF FIGURES

**FIG 1** Microarray results showing % distribution of various categories of genes that were differentially expressed in CUR treated *C. albicans*. Gene names in bold type indicate upregulation in response to CUR treatment, while those in gray type indicate downregulation.

**FIG 2** (A) Time-kill curve of CUR in combination with CWP agents; 173.7 µM CUR (63.98 µg/ml) + 1.95 µM (1.79 µg/ml) of CFW, 173.7 µM (63.98 µg/ml) CUR + 1.90 µM (1.36 µg/ml) CR and 86.8 µM (31.97 µg/ml) CUR + 31.25 µM (9 µg/ml) SDS. The x-axis is time in hours and the y-axis is Log CFU/mL. (B) % growth of *C. albicans* at above mentioned synergy concentration of CUR with CWP agents. (C) Solid medium spotting assay. The wild type (SC5314) strain was tested by spotting decreasing cell counts on YPD agar plates with CUR and CWP agents alone and in combinations.

**FIG 3** (A) Confocal microscopy analysis of membrane permeabilization assay by PI uptake. *C. albicans* was incubated with CUR alone (251 µM (92.45 µg/ml) and 502 µM (184.9 µg/ml)) and at synergy concentration with CWP agents (1.95 µM (1.79 µg/ml) CFW + 173.7 µM (63.98 µg/ml) CUR, 1.95 µM (1.36 µg/ml) CR + 173.7 µM (63.98 µg/ml) CUR, 31.25 µM (9 µg/ml) SDS + 86.8 µM (31.97 µg/ml) CUR) for 4 h.

**FIG 4** Scanning electron micrographs (SEM) showing corrugation of cell surface, squeezing of cell, and leak out of cytoplasmic content due to cell wall damage (arrow) in *C. albicans* after 24 h treatment of CUR with CWP agents at synergy concentration. (A) SEM of untreated and treated *C. albicans* with CUR (695 µM; 256 µg/ml) for 24 h showing corrugation of cell wall and squeezing of cells. (B-i), (C-i), (D-i)-Control: cells are intact and evenly shaped; (B-ii)-After treatment with 1.95 µM (1.79 µg/ml) CFW, (B-iii) 173.7 µM (63.98 µg/ml) CUR, (B-iv) 1.95 µM (1.79 µg/ml) CFW+ 173.7 µM (63.98 µg/ml) CUR; (C-ii)-After incubation with 1.90
μM (1.36 µg/ml) CR , (C-iii) 173.7 μM (63.98 µg/ml) CUR, (C-iv) 1.90 μM (1.36 µg/ml) CR+
173.7 μM (63.98 µg/ml) CUR; (D-ii) while cells incubated with 31.25 μM SDS, (D-iii) 86.8 μM
CUR, (C-iv) 31.25 μM (9 µg/ml) SDS+ 86.8 μM (31.97 µg/ml) CUR.

**FIG 5** Calcineurin, MAP kinase and stress mutants showed enhanced fungicidal activity of CUR
in *C. albicans*. Cells were grown overnight in YPD at 30°C, 5-fold serially diluted, and spotted
onto YPD medium containing CFW and CUR at the concentrations indicated. The plates were
incubated at 30°C for 48 h.

**FIG 6** Summary of altered mechanisms in *C. albicans* cells after CUR treatment. These
mechanisms are crucial for the development of drug tolerance and virulence in *C. albicans*.
FIG 1 Microarray results showing % distribution of various categories of genes that were differentially expressed in CUR treated *C. albicans*. Gene names in bold type indicate upregulation in response to CUR treatment, while those in gray type indicate downregulation.
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FIG 3 (A) Confocal microscopy analysis of membrane permeabilization assay by PI uptake. *C. albicans* was incubated with CUR alone (251 µM (92.45 µg/ml) and 502 µM (184.9 µg/ml)) and at synergy concentration with CWP agents (1.95 µM (1.79 µg/ml) CFW + 173.7 µM (63.98 µg/ml) CUR, 1.95 µM (1.36 µg/ml) CR + 173.7 µM (63.98 µg/ml) CUR, 31.25 µM (9 µg/ml) SDS + 86.8 µM (31.97 µg/ml) CUR) for 4 h.
Figure 4

Scanning electron micrographs (SEM) showing corrugation of cell surface, squeezing of cell, and leak out of cytoplasmic content due to cell wall damage (arrow) in *C. albicans* after 24 h treatment of CUR with CWP agents at synergy concentration. (A) SEM of untreated and treated *C. albicans* with CUR (695 µM; 256 µg/ml) for 24 h showing corrugation of cell wall and squeezing of cells. (B-i), (C-i), (D-i)-Control: cells are intact and evenly shaped; (B-ii) After treatment with 1.95 µM (1.79 µg/ml) CFW, (B-iii) 173.7 µM (63.98 µg/ml) CUR, (B-iv) 1.95 µM (1.79 µg/ml) CFW+ 173.7 µM (63.98 µg/ml) CUR; (C-ii)-After incubation with 1.90 µM (1.36 µg/ml) CR, (C-iii) 173.7 µM (63.98 µg/ml) CUR, (C-iv) 1.90 µM (1.36 µg/ml) CR+ 173.7 µM (63.98 µg/ml) CUR; (D-ii) while cells incubated with 31.25 µM SDS, (D-iii) 86.8 µM CUR, (C-iv) 31.25 µM (9 µg/ml) SDS+ 86.8 µM (31.97 µg/ml) CUR.

**FIG 4** Scanning electron micrographs (SEM) showing corrugation of cell surface, squeezing of cell, and leak out of cytoplasmic content due to cell wall damage (arrow) in *C. albicans* after 24 h treatment of CUR with CWP agents at synergy concentration. (A) SEM of untreated and treated *C. albicans* with CUR (695 µM; 256 µg/ml) for 24 h showing corrugation of cell wall and squeezing of cells. (B-i), (C-i), (D-i)-Control: cells are intact and evenly shaped; (B-ii) After treatment with 1.95 µM (1.79 µg/ml) CFW, (B-iii) 173.7 µM (63.98 µg/ml) CUR, (B-iv) 1.95 µM (1.79 µg/ml) CFW+ 173.7 µM (63.98 µg/ml) CUR; (C-ii)-After incubation with 1.90 µM (1.36 µg/ml) CR, (C-iii) 173.7 µM (63.98 µg/ml) CUR, (C-iv) 1.90 µM (1.36 µg/ml) CR+ 173.7 µM (63.98 µg/ml) CUR; (D-ii) while cells incubated with 31.25 µM SDS, (D-iii) 86.8 µM CUR, (C-iv) 31.25 µM (9 µg/ml) SDS+ 86.8 µM (31.97 µg/ml) CUR.
Calcineurin, MAP kinase and stress mutants showed enhanced fungicidal activity of CUR in \textit{C. albicans}. Cells were grown overnight in YPD at 30°C, 5-fold serially diluted, and spotted onto YPD medium containing CFW and CUR at the concentrations indicated. The plates were incubated at 30°C for 48 h.
FIG 6 Summary of altered mechanisms in *C. albicans* cells after CUR treatment. These mechanisms are crucial for the development of drug tolerance and virulence in *C. albicans*. 