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Cell Wall Perturbation Sensitizes Fungi to the Antimalarial Drug Chloroquine

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Chloroquine (CQ) has been a mainstay of antimalarial drug treatment for several decades. Additional therapeutic actions of CQ have been described, including some reports of fungal inhibition. Here we investigated the action of CQ in fungi, including the yeast model *Saccharomyces cerevisiae*. A genomewide yeast deletion strain collection was screened against CQ, revealing that *bck1*Δ and *slt2*Δ mutants of the cell wall integrity pathway are CQ hypersensitive. This phenotype was rescued with sorbitol, consistent with cell wall involvement. The cell wall-targeting agent caffeine caused hypersensitivity to CQ, as did cell wall perturbation by sonication. The phenotypes were not caused by CQ-induced changes to cell wall components. Instead, CQ accumulated to higher levels in cells with perturbed cell walls: CQ uptake was 2- to 3-fold greater in *bck1*Δ and *slt2*Δ mutants than in wild-type yeast. CQ toxicity was synergistic with that of the major cell wall-targeting antifungal drug, caspofungin. The MIC of caspofungin against the yeast pathogen *Candida albicans* was decreased 2-fold by 250 μM CQ and up to 8-fold at higher CQ concentrations. Similar effects were seen in *Candida glabrata* and *Aspergillus fumigatus*. The results show that the cell wall is critical for CQ resistance in fungi and suggest that combination treatments with cell wall-targeting drugs could have potential for antifungal treatment.

Fungal infections continue to represent a serious challenge to human health, due partly to interventions or other diseases that may facilitate fungal proliferation. Patients with debilitating diseases such as HIV, organ transplant recipients, major burn patients, and those treated with corticosteroids or other immunosuppressants are more susceptible to fungal infections. Among the most important opportunistic fungal pathogens are *Candida* spp., the fourth most common cause of nosocomial infection, with a case rate of 72.8 per million people per year and a mortality rate close to 34% (1). Other fungal genera that are common pathogens include *Aspergillus*, *Cryptococcus*, and *Fusarium*. Mortality rates for infections with these fungi are as high as 62% (2). Drugs used to treat fungal infections include the polyenes, azoles, and echinocandins. However, with the limited number of antifungals available, newer treatments are required. Combination treatments with antifungals have attracted considerable attention as a method of management, due to a paucity of newly emerging agents. An advantage of such combinations is that they reduce the likelihood of resistance (3).

One factor that helps in the development of novel antifungal entities or in elucidating modes of action is that many fungal genome sequences are now available. Certain fungi are also highly amenable to laboratory manipulation. Thus, the yeast *Saccharomyces cerevisiae* is broadly adopted as a eukaryotic cell model of choice. The yeast model has been applied to characterize the actions of antifungal drugs (4, 5) as well as a diverse range of other therapeutic compounds, including antimalarial drugs (6, 7). Antimalarials are notoriously poorly characterized in regard both to their modes of action against the malaria parasite and to the adverse reactions that many provoke in humans. A screen of a yeast deletion strain collection against the antimalarial drug quinine revealed a novel mode of quinine action (6). Mutants defective for biosynthesis of the amino acid tryptophan were quinine hypersensitive. Further experiments revealed that quinine competed with tryptophan for uptake via the Tat2p transporter, leading to tryptophan starvation, suggesting a novel mechanism of quinine toxicity. Moreover, the power of the yeast model was exemplified by a recent extrapolation of these findings to malaria patients in hospitals (8). These clinical data indicate that quinine also competes with tryptophan in humans and that dietary tryptophan may suppress adverse reactions of patients to quinine.

The antimalarial drug chloroquine (CQ) is chemically distinct from the structural relationship between quinine and tryptophan (6). Despite being an older drug, CQ is safe and inexpensive and remains a recommended antimalarial in areas affected by CQ-sensitive malarial infections, particularly by *Plasmodium vivax* (9). The mode of CQ action has been attributed to binding of the drug to heme in the parasite food vacuole, resulting in decreased heme polymerization; free heme is toxic to the parasite. CQ may also increase the pH of the parasite digestive vacuole or inhibit an endogenous function through binding to the PfCRT protein (10). In addition to this antimalarial activity, CQ has been shown to have anti-inflammatory properties and has been used widely in the treatment of arthritis (11). There have also been reports of CQ activity against fungal pathogens (12–15). The mechanism is thought to involve alkalinization of the host environment of the

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fungi, with associated iron deprivation in some cases. CQ has also been shown to inhibit thiamine transport in yeast as well as human cells (16).

The objective at the outset of this study was to apply the yeast tool to gain new insights into chloroquine action. The cell wall integrity pathway genes *SLT2* and *BCK1* were characterized as key determinants of CQ resistance. With the aim of explaining this result, we showed that cell wall perturbation produces CQ hypersensitivity due to elevated CQ uptake. Because the cell wall is the target of existing antifungal drugs, our final aim was to investigate the possibility of combining such drugs with CQ to give a synergistic antifungal action.

MATERIALS AND METHODS

Yeast strains, deletion strain screen, and growth assays. BY4743 was the strain background used in experiments involving *Saccharomyces cerevisiae*, except where stated otherwise. BY4743 and isogenic homozygous deletion strains were obtained from Euroscarf (Frankfurt, Germany). Additional fungi used in this study were *Candida albicans* SC5314, *Candida glabrata* BG2, and *Aspergillus fumigatus* AF293. *S. cerevisiae* YPH499 and the isogenic mutant YMS348s (*chs1*Δ *chs2*Δ *chs3*Δ) were kind gifts from Martin Schmidt, Des Moines University (17). Deletion strain screens were performed by replica inoculation of strains into yeast extract-peptone-dextrose (YEPD) broth in a 96-well format, either supplemented or not with 2.9 mM chloroquine diphosphate (Sigma). The screen and calculation of growth ratios were performed as described previously (6). Briefly, growth ratios for each strain were calculated by dividing the optical densities at 600 nm (OD_{600}) obtained under control conditions (minus CQ) by those for parallel CQ-supplemented incubations. Strains with a mean growth ratio of ≥1.45 ($n = 2$) from the initial screen were rearrayed onto new 96-well plates and screened three more times in duplicate. Strains giving a median growth ratio across all screens of ≥1.45 were deemed to be CQ hypersensitive. For other growth experiments, overnight cultures in YEPD broth were diluted in fresh medium (10 ml in 50-ml flasks) and cultured with orbital shaking to an OD_{600} of ~2.0. For spotting assays, the cultures were serially diluted 1:10 with phosphate-buffered saline (PBS) and spotted (5 μl) onto YEPD agar (18) supplemented as indicated with chloroquine, caffeine (Sigma), calcofluor white (CW; Sigma), or sorbitol. Plates were observed after incubation at 30°C for 48 h.

Etest strips containing caspofungin (CSP) were used to assay simultaneous treatments with chloroquine or related drugs and caspofungin. After subculturing into YEPD broth and incubation for 3 to 4 h, to ~2×10^6 CFU ml^{-1}, organisms were spread with a sterile cotton swab to cover the surface of RPMI-2G agar (RPMI 1640 medium supplemented with 2% [wt/vol] glucose and 1.5% [wt/vol] agar and buffered with 0.165 M MOPS [morpholinepropanesulfonic acid; Sigma] adjusted to pH 7 with sodium hydroxide). The agar was supplemented with amodiaquine, chloroquine, mefloquine, quinacrine, or quinine, supplied at subinhibitory concentrations. Etest strips containing caspofungin (bioMérieux) were placed aseptically onto the inoculated agar. Plates were incubated for 48 h at 30°C (*S. cerevisiae*) or 37°C before examination.

Cell wall stress assay. Sonication was used to stress yeast cell walls as described previously (19). Strains were cultured overnight in the absence or presence of CQ or caffeine. The cells were then subcultured into fresh medium supplemented as described above and were incubated for 3 h, to an OD_{600} of ~1.0. Aliquots (1 ml) of cell suspension were transferred to microcentrifuge tubes. Where specified, cells were washed twice with PBS to remove drugs before sonication. Samples were sonicated for 1 min at 70% power, using a sonicator (ColePalmer) equipped with a 3-mm-diameter probe. Samples (100 μl) were removed before and after sonication, diluted with PBS, and spread plated on YEPD agar. Percent viability was determined from CFU counts after incubation at 30°C for 2 days, as described previously (20). All statistical analyses were performed with Statistical Package for the Social Sciences (SPSS) software, version 17.

Determination of cell wall components. β-1,3-Glucan was probed with aniline blue as described previously (21). Cells were cultured with or without CQ either overnight or for 3 h, to an OD_{600} of 0.5 to 0.8, and then diluted to 2.5×10^6 cells ml^{-1}. Cells were washed with TE buffer (10 mM Tris, 1 mM EDTA, adjusted to pH 8 with HCl) and suspended in 250 μl of the same buffer. NaOH was added to a final concentration of 1 M before incubation at 80°C for 30 min. A 1.05-ml volume of aniline blue mixture (0.03% [wt/vol] aniline blue [Sigma], 0.18 M HCl, 0.49 M glycine-NaOH, pH 9.5) was added. Samples were vortexed and incubated at 50°C for 30 min. After a further 30 min at room temperature, the amount of β-1,3-glucan was estimated by fluorescence spectrophotometry (Cary Eclipse Varian), with a λ_{ex} value of 400 nm and a λ_{em} value of 460 nm.

The mannosylphosphate component of cell wall mannoprotein was determined by alcian blue staining as described previously (22). Cells were cultured with or without CQ either overnight or for 3 h, to an OD_{600} of ~1.0. Samples (5 ml) were washed twice with 2 ml 0.02 M HCl and resuspended in 1 ml of 0.005% (wt/vol) alcian blue in 0.02 M HCl. The mixture was left to stand at room temperature for 10 min and then centrifuged for 3 min at 18,000 × *g*. Unbound alcian blue was determined from the OD_{600} of the supernatant, with reference to a standard curve prepared with alcian blue solutions ranging from 0 to 0.05% (wt/vol) in 0.02 M HCl. The amount of alcian blue bound to cells was then calculated by subtracting the amount of unbound dye from the starting amount of 50 μg alcian blue.

The chitin content of cell walls was probed with calcofluor white (23). Cells were cultured with or without CQ either overnight or for 3 h and then adjusted to 1×10^6 CFU ml^{-1}, washed twice with PBS, and stained with 1.1 μM calcofluor white for 30 min. Stained cells were washed and resuspended in 1 ml PBS. Calcofluor white fluorescence was quantified with a Becton, Dickinson LSR Flow II flow cytometer equipped with a 365-nm laser. Calcofluor white was detected with a 440/40BP emission filter.

Chloroquine uptake. Chloroquine uptake by cells was estimated using a fluorescently labeled chloroquine molecule, LynxTag-CQ green (BioLynx Technologies). Aliquots (100 μl) of cells grown to an OD_{600} of ~2.0 in YEPD broth were transferred to microcentrifuge tubes. Chloroquine was added together with 2 μl of 1 mM LynxTag-CQ green and, where specified, caffeine. Cells were incubated in the dark with shaking at 30°C. Samples (10 μl) were removed at intervals, and cells were washed and resuspended in a 1:100 dilution of PBS. Fluorescence from cellular LynxTag-CQ green was measured with a Becton, Dickinson FACSCanto flow cytometer, with excitation at 488 nm and emission detected through a 505LP, 530/30BP filter. A 10-μl sample was also washed and examined microscopically with a 100× oil immersion lens and appropriate filters to confirm the presence of CQ within yeast cells.

RESULTS

Deletion strains with altered chloroquine resistance. The yeast homozygous diploid deletion mutant collection was screened to identify genes that are important for CQ resistance. Preliminary experiments showed that 2.9 mM CQ was just sufficient to exert a mild (~10%) slowing of wild-type growth, and this concentration was selected for screening. A total of 97 CQ-sensitive strains (growth ratios of ≥1.45) were identified from the genomewide screen, and 23 of these phenotypes were subsequently confirmed in specific tests of the 97 putative strains (see Table S1 in the supplemental material). The 23 deleted genes of the sensitive strains were grouped into functional categories (24), and the resultant distributions were analyzed for significant differences compared with genomewide distributions for *S. cerevisiae*. "Stress response" was the most highly overrepresented functional category in the annotations of genes in the CQ-sensitive data set, owing to the sensitivity of deletion strains such as the *sat4*Δ/*sat4*Δ, *slt2*Δ/*slt2*Δ, and *bck1*Δ/*bck1*Δ strains (Table 1). The evident re-

TABLE 1 Overrepresentation of specific functional categories in annotations of genes from the chloroquine-sensitive data set

Functional category[a,b]	*P* value[c]	Genes
Stress response	1.51×10^{-3}	*YBR016W*, *SAT4*, *SLT2*, *BCK1*
Modification by phosphorylation, dephosphorylation, or autophosphorylation	2.51×10^{-3}	*SAT4*, *SLT2*, *BCK1*, *PTC5*
MAPKKK cascade	3.22×10^{-3}	*SLT2*, *BCK1*
Directional cell growth (morphogenesis)	4.51×10^{-3}	*SLT2*, *BCK1*
Vacuole or lysosome	8.41×10^{-3}	*VPS41*, *TLG2*

[a] The data set comprises the defective gene functions of each deletion strain that scored chloroquine sensitive.
[b] All functional categories scoring *P* values of ≤0.01 are shown.
[c] According to http://funspec.med.utoronto.ca/.

quirement for *SLT2* and *BCK1* in normal CQ resistance yielded additional overrepresented categories, including the mitogen-activated protein kinase kinase kinase (MAPKKK) cascade and directional cell growth.

CQ sensitivity of cell wall integrity pathway mutants is specific to the *bck1*Δ and *slt2*Δ mutations and to drug. Bck1p and Slt2p have key roles in the cell wall integrity pathway (25–27). We conducted specific CQ resistance tests with other mutants of the pathway. These were not detected in the above screen (Table 1), but the screening criteria were stringent to avoid false-positive results (6). Whereas the *bck1*Δ/*bck1*Δ and *slt2*Δ/*slt2*Δ mutants showed marked growth defects versus the wild type in the presence of CQ (Fig. 1), any effects in other Slt2 pathway mutants were slight (see Fig. S1A in the supplemental material). (Note that particularly high CQ concentrations [>10 mM] were required in certain of these experiments to overcome the CQ resistance of wild-type *S. cerevisiae*; the relevant MICs were ~20, 1.5, and 1.5 mM CQ for the wild-type, *bck1*Δ/*bck1*Δ, and *slt2*Δ/*slt2*Δ strains, respectively). Similarly, deletion strains defective for cell surface sensors that signal to the Slt2 pathway were not markedly CQ sensitive (see Fig. S1B in the supplemental material). There is some redundancy within the cell wall integrity pathway (25, 27), and the results indicate that a noncompensatable defect in the pathway (brought about only by *BCK1* or *SLT2* deletion) is required to elicit the CQ sensitivity phenotype.

The effect of *BCK1* or *SLT2* deletion on drug sensitivity was tested with other quinoline-containing antimalarials: quinine, mefloquine, amodiaquine, and quinacrine. Unlike CQ, none of these other drugs revealed a marked hypersensitivity phenotype of the mutants versus the wild type (Fig. 1). The mutants exhibited a very slight sensitivity to quinacrine. The results highlight the CQ specificity of the phenotypes.

Cell wall damage and chloroquine resistance. Deletion of *BCK1* or *SLT2* in *S. cerevisiae*, as well as in pathogens such as *Candida* spp., has been reported to yield sensitivity to cell wall-damaging agents such as caffeine and calcofluor white (CW) (28, 29). To corroborate an involvement of cell wall integrity in CQ action, we tested the effects of caffeine and CW on CQ resistance. The *bck1*Δ/*bck1*Δ and *slt2*Δ/*slt2*Δ mutants were confirmed to be caffeine and CW hypersensitive (Fig. 2A). Consistent with cell wall damage, this sensitivity was rescued with 1 M sorbitol, and this was also the case for CQ (Fig. 2A). CQ was supplied in combination with CW or caffeine at concentrations that were just subinhibitory to the relevant yeast strains when each drug was supplied alone. CW did not cause hypersensitivity to CQ (Fig. 2B). However, a combination of CQ and caffeine gave markedly greater growth inhibition than the individual effects of the two drugs. This effect was apparent in the wild-type, *bck1*Δ/*bck1*Δ, and *slt2*Δ/*slt2*Δ strains. This indication of some synergy in the effects of CQ and caffeine suggested that these drugs may have different molecular targets but their actions involve a common metabolic product or cell structure.

To substantiate that CQ action is related to cell wall integrity, cells were sonicated during CQ exposure. Sonication physically weakens the yeast cell wall and makes it more susceptible to the actions of chemical cell wall stressors (19). Wild-type cells were cultured with different subinhibitory concentrations of caffeine or CQ, and viability was determined before and after sonication for 1 min in the presence of caffeine or CQ. In controls where drugs were absent, the sonication treatment had a negligible effect on viability (Fig. 3). However, in the presence of 4 mM caffeine or CQ, viability was decreased ~80% by sonication. This was consistent with CQ action being related to cell wall integrity.

CQ toxicity is not mediated by effects on cell wall composition. We considered two hypotheses to explain the above results: (i) the mode of CQ action against yeast involves targeting of the cell wall, similar to the case for CW and caffeine; and (ii) cell wall

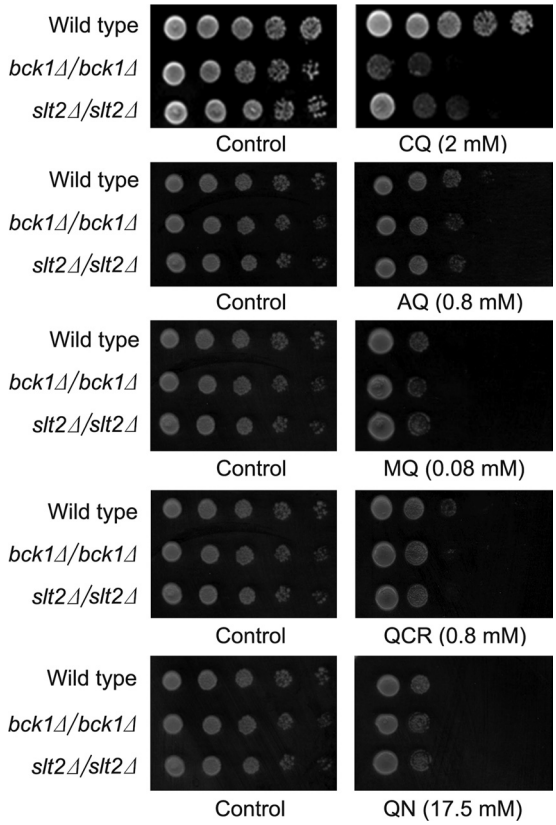

FIG 1 Chloroquine sensitivity of strains defective for *BCK1* or *SLT2*. Exponential-phase cultures of *S. cerevisiae* BY4743 and isogenic *bck1*Δ/*bck1*Δ and *slt2*Δ/*slt2*Δ strains were serially diluted and spotted onto agar supplemented or not with drugs at the indicated concentrations. Images were captured after incubation for 48 h at 30°C. CQ, chloroquine; AQ, amodiaquine; MQ, mefloquine; QCR, quinacrine; QN, quinine.

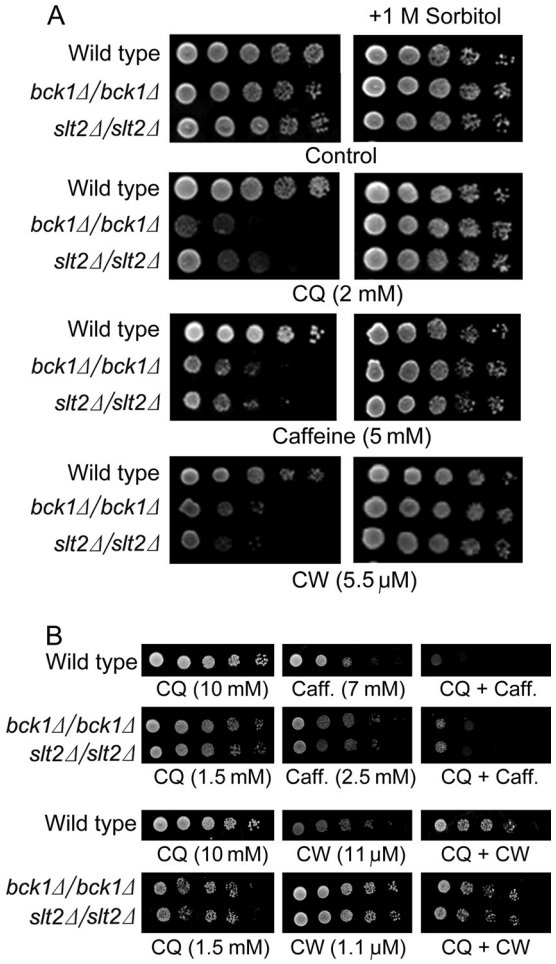

FIG 2 Rescue of chloroquine sensitivity by sorbitol and synergistic action with caffeine. (A) Exponential-phase cultures of *S. cerevisiae* BY4743 (wild type) and isogenic *bck1*Δ/*bck1*Δ and *slt2*Δ/*slt2*Δ strains were serially diluted and spotted onto agar supplemented or not with CQ, caffeine, or calcofluor white (CW) at the indicated concentrations and in the absence (left) or presence (right) of 1 M sorbitol. (B) Strains were spotted onto agar supplemented with the agents at concentrations that were just subinhibitory to the relevant strain(s) when supplied singly. The same concentrations were used in combinations of the agents. All images were captured after incubation for 48 h at 30°C.

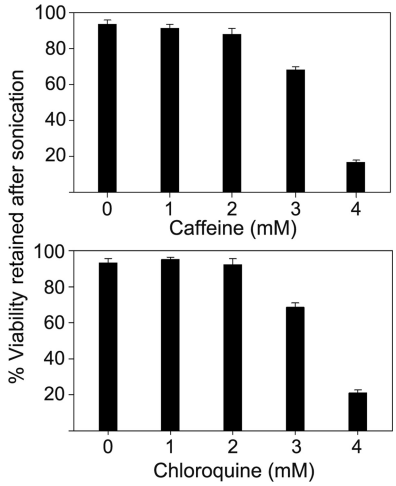

FIG 3 Sonication sensitizes cells to chloroquine. Overnight cultures of *S. cerevisiae* BY4743 supplemented with the indicated concentrations of caffeine or chloroquine were sonicated for 1 min. Percent viability was calculated from CFU counts determined after sonication with reference to corresponding counts before sonication (without sonication, neither caffeine nor chloroquine affected viability at the indicated concentrations; sonication alone did not significantly affect viability). Means ± standard errors of the means (SEM) are shown for three independent determinations.

integrity is important for CQ resistance, possibly by preventing intracellular access of the drug. To give clues to any specific cell wall components that may be affected by CQ (hypothesis i), we first conducted specific CQ resistance assays with 70 mutants defective for biosynthesis of different cell wall components, including β-1,3-glucan, β-1,6-glucan, mannoprotein, and chitin. These mutants were not detected as CQ sensitive in the screen (see Table S1 in the supplemental material), nor did they prove to be CQ sensitive in our specific assays (data not shown). This suggested that CQ does not target a particular cell wall component. To corroborate this, we assayed each of the major cell wall components of *S. cerevisiae*, exposed or not to CQ. The outcomes described below did not differ whether cells were incubated for 3 h or overnight in the presence of CQ before cell wall analysis. Aniline blue staining was used to indicate the β-1,3-glucan content (21), which did not differ significantly between CQ-exposed and nonexposed cells (see Fig. S2A in the supplemental material). Similar results were obtained for the mannosylphosphate component of cell wall mannoprotein (see Fig. S2B in the supplemental material), according to alcian blue staining (22). However, using a subinhibitory concentration of 1.1 μM CW in order to stain chitin (23, 30), we noted a >1.5-fold increase in the apparent chitin composition of the cell walls of CQ-treated cells (Fig. 4A) ($P \leq 0.0002$ at all CQ concentrations versus the CQ-free control). To test whether increased chitin content may be a cause of CQ toxicity, a chitin-defective triple chitin synthase mutant, YMS348s (*chs1*Δ *chs2*Δ *chs3*Δ), was examined for CQ resistance. First, it was confirmed that the mutant was resistant to normally inhibitory concentrations of CW (Fig. 4B), consistent with a mode of CW action involving binding to nascent chitin fibrils (31, 32). In contrast, the YMS348s mutant exhibited a CQ resistance similar to that of the isogenic wild type, indicating that (increased) chitin is not an important mode of CQ action (Fig. 4B). We confirmed that there was no CQ-dependent increase in chitin content in the YMS348s mutant.

Cell wall perturbation facilitates CQ uptake into yeast cells. As the above data did not support the hypothesis that CQ causes cell wall damage, we considered the alternative hypothesis (hypothesis ii) that cell wall integrity is required for normal CQ resistance. Therefore, we performed sonication assays similar to those shown in Fig. 3, but with the drug removed just prior to sonication. We reasoned that if cell wall damage caused by (prior) CQ exposure was the reason for sonication sensitivity (Fig. 3), then cells should still exhibit such sensitivity for some time after removal of CQ. In contrast, the continued presence of CQ would be required for sonication sensitivity if the relevant effect was to enable CQ entry into the cell. In the case of caffeine as a positive control, viability was decreased ≥80% due to sonication, whether 4 mM caffeine was retained (Fig. 3) or removed just prior to sonication (Fig. 5, top panel) (sonication without any caffeine treat-

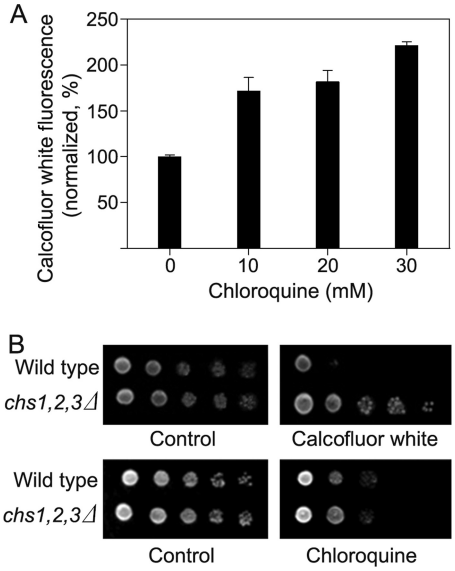

FIG 4 Chloroquine-induced increases in cell wall chitin are not a cause of chloroquine toxicity. (A) Cultures of *S. cerevisiae* BY4743 were incubated for 3 h with the indicated concentrations of chloroquine and then probed for chitin content with 1.1 μM CW (this concentration is noninhibitory to BY4743). Mean values ± SEM are shown for three independent determinations. (B) Exponential-phase cultures of *S. cerevisiae* YPH499 (wild type) and a chitin synthase-defective derivative strain, YMS348s (*chs1*Δ *chs2*Δ *chs3*Δ), were serially diluted and spotted onto agar supplemented or not with 22 μM CW or 25 mM CQ. Images were captured after incubation for 48 h at 30°C.

ment had a negligible effect on viability). In contrast, cells were relatively resistant to sonication after growth in up to 10 mM CQ when the drug was removed before sonication (Fig. 5, bottom panel), whereas the presence of 4 mM CQ during sonication produced an ~80% loss of viability (Fig. 3). These outcomes for CQ were consistent with the hypothesis that normal cell wall integrity helps to prevent CQ entry and its resultant toxicity in cells. To test that hypothesis directly, uptake of the drug was examined by use of a fluorescent probe (33). LynxTag-CQ green uptake was approximately 2-fold and 3-fold higher in the *bck1*Δ/*bck1*Δ and *slt2*Δ/*slt2*Δ mutants, respectively, than in wild-type *S. cerevisiae* (Fig. 6A). This was in keeping with these mutants' CQ sensitivities. Similarly, other treatments that were used to perturb the cell wall (caffeine treatment and sonication) and which sensitized cells to CQ were also associated with increased LynxTag-CQ green uptake (Fig. 6B and C). The results indicate that CQ toxicity is greater in cell wall-perturbed cells due to increased CQ uptake.

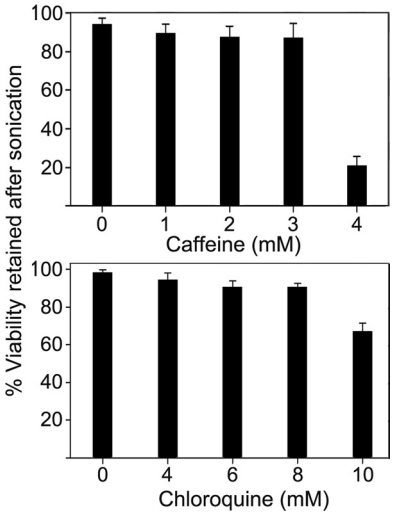

FIG 5 Sonication hypersensitivity requires the presence of chloroquine during sonication. Overnight cultures of *S. cerevisiae* BY4743 supplemented with the indicated concentrations of caffeine or chloroquine were washed to remove the drug and then sonicated for 1 min. Percent viability was calculated from CFU counts determined after sonication with reference to corresponding counts before sonication (without sonication, neither caffeine nor chloroquine affected viability at the indicated concentrations; sonication without prior drug exposure did not significantly affect viability). Mean values ± SEM are shown for three independent determinations.

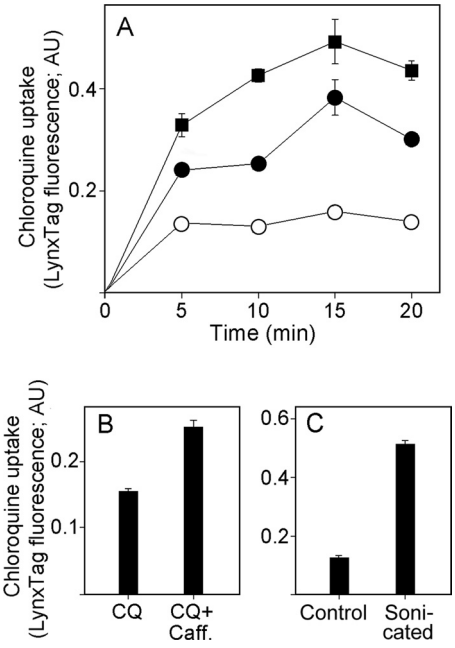

FIG 6 Cells with perturbed cell walls accumulate larger amounts of chloroquine. (A) Exponential-phase cultures of *S. cerevisiae* BY4743 (○) and isogenic *bck1*Δ/*bck1*Δ (●) and *slt2*Δ/*slt2*Δ (■) strains were incubated in the presence of 0.4 mM chloroquine spiked with 20 μM LynxTag-CQ green. The fluorescence of cellular LynxTag-CQ green was determined at intervals by flow cytometry. (B) LynxTag-CQ green uptake was determined in BY4743 cells after incubation for 3 h with 1 mM CQ or 1 mM CQ plus 1 mM caffeine. (C) LynxTag-CQ green uptake was determined in BY4743 cells during incubation with 4 mM CQ, before or after sonication for 1 min. All values are means ± SEM for three independent determinations. AU, arbitrary units.

Synergistic activity of CSP and chloroquine. The dependency of chloroquine resistance on yeast cell wall integrity suggested the possibility that chloroquine could have a synergistic therapeutic action with a cell wall-targeting antifungal drug. The echinocandin drug caspofungin (CSP) and CQ were examined for synergistic effects on pathogenic fungi, using CSP Etest strips as applied clinically and following the guidelines for synergy determination (34). In *Candida albicans*, there was evidence for marked synergy with subinhibitory concentrations of CQ (Fig. 7). For example, simultaneous exposure to 2 mM CQ decreased the MIC of CSP approximately 3-fold. In contrast, only additive effects were observed with subinhibitory concentrations of other quinolone-containing drugs (Table 2). The above analyses also inferred a marked drop in the MIC of CQ in the presence of CSP, where ~20

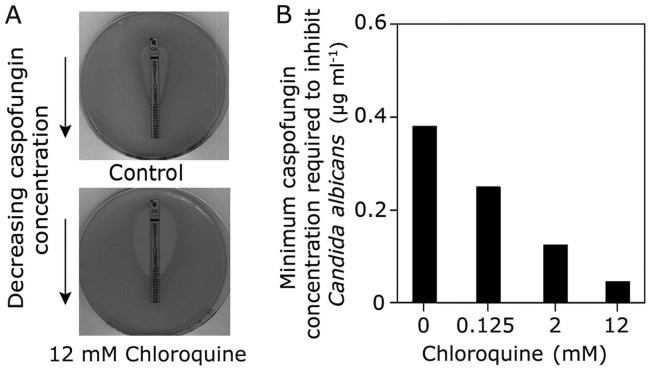

FIG 7 Synergistic action of caspofungin and chloroquine against *Candida albicans*. (A) *C. albicans* SC5314 was spread plated on RPMI-2G agar and overlaid with an Etest caspofungin strip. Images of growth were captured after 48 h of incubation at 37°C. (B) The MIC of caspofungin was determined from Etest analysis (A) on agar supplemented with different subinhibitory concentrations of chloroquine. Results from triplicate determinations were identical, so error bars are not shown. Synergy was defined as a decrease of ≥3 dilutions, according to Etest, in the combination MIC compared to caspofungin alone. Additivity (or indifference) was defined as a decrease of <3 dilutions (34, 50). When supplied alone, CQ was noninhibitory at all indicated concentrations.

mM CQ was required for growth inhibition in the absence of CSP but only ~0.125 mM CQ inhibited growth at 0.25 μg ml^{-1} CSP. CQ and CSP also exhibited synergistic action against *C. glabrata*. Although the effect was less marked than in the *Candida* spp., the MIC of CSP was also decreased by the presence of CQ in the CQ-sensitive filamentous pathogen *Aspergillus fumigatus*. The data indicate that the antifungal action of CSP is increased by the presence of CQ and *vice versa*. The fact that these effects of CQ were evident among several fungi with differing cell wall compositions (35) was in keeping with the observation that the overall integrity of the cell wall, rather than particular cell wall components, determines CQ resistance (see "CQ toxicity is not mediated by effects on cell wall composition").

DISCUSSION

S. cerevisiae was used successfully as a tool in this study to show how fungi with perturbed cell walls are hypersensitized to chloroquine. The fungal cell wall has been a long-standing target for development of other antifungal drugs. The echinocandin antifungals target one of the main cell wall components, β-1,3-glucan, and are used to treat invasive fungal infections by *Candida* and *Aspergillus* spp. (36, 37). In this study, the CQ hypersensitivity of the *bck1*Δ/*bck1*Δ and *slt2*Δ/*slt2*Δ yeast deletion mutants highlighted a role for the MAPK cell wall integrity pathway (25–27). This was specific to CQ resistance versus resistance to other closely related quinoline-containing antimalarials. One structural difference between the drugs that may be pertinent here is the occurrence of an aryl side chain in quinine, amodiaquine, and mefloquine, whereas CQ and quinacrine have an alkyl side chain (38). Since quinacrine was the only drug tested other than CQ for which there was a suggestion of sensitivity in the *bck1*Δ/*bck1*Δ and *slt2*Δ/*slt2*Δ mutants, the alkyl side chain may be one structural feature that helps to determine this phenotype. A key difference is that quinacrine has three fused aromatic rings, whereas CQ has two rings.

Agents that target a component of the fungal cell wall typically cause altered levels of that component. The content of other cell wall components is commonly modified in compensation to help sustain cell wall strength and integrity (39). The fact that mutants of cell wall components were not CQ hypersensitive was consistent with the cell wall not being the primary target of CQ action. There were increases in the chitin content of CQ-exposed cells, but chitin was not a target of CQ action, as CQ resistance was not affected in chitin synthase-defective cells. This contrasted with the case for calcofluor white, which interferes with chitin formation as its mode of action and causes increased chitin synthase activity (31, 32, 40, 41). The increased chitin content seen with CQ appeared to be an incidental effect, consistent with the fact that the cell wall acts to help block CQ uptake rather than be targeted by CQ action.

Little is known about the uptake of quinolone-containing drugs into yeast cells. However, a mode of antifungal CQ action based on alkalinization of the host environment and iron deprivation has been proposed (12, 14, 15). Pathogens such as *C. albicans* themselves can actively alkalinize their environments (42), and this action could exacerbate CQ action. Related mechanisms of CQ action are known for *Plasmodium* spp. (10). Recently, CQ was shown to inhibit thiamine transporters in both yeast and human cells (16). Our study indicates that the fungal cell wall acts as a barrier to help preclude the toxic action(s) of CQ and therefore that the potential of CQ as an antifungal agent (12–15) is enhanced considerably by cell wall perturbation.

The potential value of drug combinations for the management

TABLE 2 Antifungal Etest assays of caspofungin and chloroquine drug combinations

Organism	Drug[a]	Concn (mM)	Caspofungin MIC[b] (μg ml^{-1})	Effect[c]
C. albicans SC5314	None	0	0.38	NA
	CQ	0.125	0.25	Additivity
		0.25	0.19	Additivity
		2	0.125	Synergy
		12	0.047	Synergy
	AQ	0.06	0.38	Additivity
		0.3	0.38	Additivity
	QN	0.4	0.38	Additivity
		2.0	0.38	Additivity
	MQ	0.01	0.38	Additivity
		0.05	0.38	Additivity
	QCR	0.05	0.25	Additivity
		0.25	0.125	Synergy
C. glabrata BG2	None	0	0.5	NA
	CQ	0.4	0.19	Synergy
		2.0	0.023	Synergy
A. fumigatus AF293	None	0	0.19	NA
	CQ	0.05	0.19	Additivity
		0.25	0.094	Additivity
S. cerevisiae BY4743	None	0	1.5	NA
	CQ	3	0.25	Synergy
		15	0.064	Synergy

[a] When supplied alone, CQ was noninhibitory at all indicated concentrations. The MICs of CQ were as follows: for *C. albicans*, 15 mM; for *C. glabrata*, 4 mM; for *A. fumigatus*, 0.5 mM; and for *S. cerevisiae*, 20 mM. CQ, chloroquine; AQ, amodiaquine; QN, quinine; MQ, mefloquine; QCR, quinacrine.
[b] Results from triplicate determinations were identical, so errors are not shown.
[c] For definitions of synergy and additivity, see the legend to Fig. 7. NA, not applicable.

of fungal infections is well documented (3). For example, cell wall-perturbing agents can be used in conjunction with antifungals that target the cell membrane, such as polyenes and triazoles, a strategy that has proven beneficial in critically ill patients (43). Drug combinations may reduce the risk of resistance and adverse effects and decrease the treatment duration. The use of one antifungal to increase penetration of another was exploited here with CQ and the cell wall-targeting echinocandin drug CSP. CSP is used to treat serious fungal infections such as those caused by *Candida* spp. (where Slt2p can determine CSP tolerance, as in *S. cerevisiae*) (44, 45), and it has been used alongside other antifungals, such as amphotericin and fluconazole (36, 37). CSP acts by targeting the enzyme complex β-1,3-glucan synthase required for β-1,3-glucan synthesis. In the present study, subinhibitory concentrations of CQ and caspofungin exhibited synergistic activity *in vitro*. This was observed in pathogenic fungi (*C. albicans*, *C. glabrata*, and *A. fumigatus*) as well as *S. cerevisiae*. The lowest concentration at which a decrease in the MIC of caspofungin was evident *in vitro* was 125 μM CQ. This concentration is higher than those typically encountered in the clinical setting. *In vivo* concentrations in the plasmas of patients treated with CQ are reported to be <5.9 μM (46). Furthermore, concentrations as low as 32 μM CQ are known to cause growth inhibition in human cell lines (47). However, whereas the concentrations used in this study were appropriate *in vitro*, a mode of antifungal CQ action that relies on iron deprivation due to external alkalinization (12, 14, 15) could be expected to be exacerbated markedly *in vivo*. Thus, fungal pathogens would likely be more susceptible to caspofungin-dependent CQ uptake during *in vivo* infection. Accordingly, the synergistic activity of CQ and caspofungin could have the potential for clinical application. A particular advantage of this possibility is that inclusion of inexpensive CQ could mean that less of the more-expensive echinocandin drug is needed for treatment. In addition, thanks to the extensive treatment of malaria patients with the drug over the last several decades, CQ is known to be relatively safe and is very well characterized for other indications. These findings could prove especially relevant to malaria patients with serious fungal infections (48, 49).

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