Cell-wall Perturbation Sensitizes Fungi to the Antimalarial Drug Chloroquine

Farida Islahudin¹,², Combiz Khozoie³, Steven Bates⁴, Kang-Nee Ting⁵, Richard J. Pleass⁶ and Simon V. Avery¹*

School of Biology, University of Nottingham, Nottingham NG7 2RD, UK

¹School of Biology, University of Nottingham, Nottingham NG7 2RD, UK

²School of Pharmacy, University of Nottingham Malaysia Campus, 43500 Semenyih, Malaysia

³Present address: Penn State University, Department of Veterinary and Biomedical Sciences, University Park, PA 16802, USA

⁴University of Exeter, College of Life & Environmental Sciences, Exeter EX4 4QD, UK

⁵School of Biomedical Sciences, University of Nottingham Malaysia Campus, 43500 Semenyih, Malaysia

⁶University of Liverpool, Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK

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*Corresponding author. Simon.Avery@nottingham.ac.uk

Tel. +44 115 9513315; Fax. +44 115 9513251
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 CQ action in fungi, including the yeast model *Saccharomyces cerevisiae*. The genome-wide yeast deletion strain collection was screened against CQ, revealing that *bck1Δ* and *slt2Δ* 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 was accumulated to higher levels in cells with perturbed cell walls; CQ uptake was two- to three-fold greater in *bck1Δ* or *slt2Δ* mutants than in wild type yeast. CQ toxicity was synergistic with that of the major cell wall-targeting antifungal drug, caspofungin. The MIC for caspofungin against the yeast pathogen *Candida albicans* was decreased two-fold by 250 µM CQ, and by up to five-fold at higher CQ concentrations. Similar effects were seen in *C. 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 burns 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 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. Recently, 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 mode 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* has become 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, with regard both to their modes of action against the malaria parasite and to the adverse reactions that many provoke in humans. A screen of the 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 indicated that quinine also competes with tryptophan in humans, and that dietary tryptophan may suppress adverse reactions of patients to quinine.

The antimalarial drug chloroquine 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 malaria infections, particularly by *Plasmodium vivax* (9). The mode of CQ action has been attributed to binding of the drug to haem in the parasite food vacuole, resulting in decreased haem polymerization. Free haem 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 widely used 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 alkalinisation of the host environment of the 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 the study was to apply the yeast tool to gain new insights to chloroquine action. The cell wall integrity pathway genes *SLT2* and *BCK1* were characterised 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. As 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 synergistic antifungal action.
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 from Euroscarf (Frankfurt, Germany). Additional fungi used in this study were *Candida albicans* SC5314, *C. glabrata* BG2 and *Aspergillus fumigatus* AF293. *S. cerevisiae* YPH499 and the isogenic mutant YMS348s (*chs1Δ,chs2Δ,chs3Δ*) were kind gifts from Dr. Martin Schmidt, Des Moines University (17). Deletion strain screens were performed by replica inoculation of strains to YEPD broth in 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 OD$_{600}$ readings obtained under control conditions (minus CQ) by those for parallel CQ-supplemented incubations. Strains with a mean growth ratio of $\geq 1.45$ (n=2) from the initial screen were re-arrayed onto new 96-well plates and screened three further times in duplicate. Strains giving a median growth ratio across all screens $\geq 1.45$ were deemed to be CQ hypersensitive. For other growth experiments, overnight cultures in YEPD broth were diluted into fresh medium (10 ml in 50 ml flasks) and cultured with orbital shaking to OD$_{600}\sim 2.0$. For spotting assays, the cultures were serially diluted 1:10 with phosphate buffered saline and spotted (5 µl) onto YEPD agar (18) supplemented as indicated with chloroquine, caffeine (Sigma), calcofluor white (Sigma) or sorbitol. Plates were observed after incubation at 30°C for 48 h.

Epsilometer test (Ettest) strips containing caspofungin were used to assay simultaneous treatments with chloroquine or related drugs and caspofungin. After sub-culturing to YEPD broth and incubation for 3-4 hours to $\sim 2 \times 10^6$ 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% (w/v) glucose, 1.5% (w/v) agar and buffered with 0.165...
M MOPS (Sigma), adjusted to pH 7 with sodium hydroxide. The agar was supplemented with amodiaquine, chloroquine, mefloquine, quinacrine or quinine, supplied at sub-inhibitory concentrations. E-test strips containing caspofungin (BioMerieux) were placed aseptically onto the inoculated agar. Plates were incubated for 48 h at 30ºC (S. cerevisiae) or 37ºC 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 sub-cultured to fresh medium supplemented as before and incubated for 3 h until OD$_{600}$~1.0. Aliquots (1 ml) of cell suspension were transferred to microfuge 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 to YEPD agar. Percentage viability was determined from colony forming unit counts after incubation at 30ºC for 2 d, 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 (21). Cells were cultured with or without CQ either overnight or for 3 h, until OD$_{600}$=0.5–0.8, then diluted to 2.5 x 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 80ºC for 30 min. A 1.05 ml volume of aniline blue mixture [0.03% (w/v) aniline blue (Sigma), 0.18 M HCl, 0.49 M glycine/NaOH, pH 9.5] was added. Samples were vortexed and incubated at 50ºC for 30 min. After a further 30 min at room temperature, β-1,3-glucan was estimated by fluorescence spectrophotometry (Cary Eclipse Varian) at λ$_{ex}$=400 nm, λ$_{em}$ =460nm.
The mannosylphosphate component of cell wall mannoprotein was determined with alcian blue staining as described previously (22). Cells were cultured with or without CQ either overnight or for 3 h, until OD$_{600}$~1.0. Samples (5 ml) were washed twice with 2 ml 0.02-M HCl and resuspended in 1 ml of 0.005% (w/v) alcian blue in 0.02 M HCl. The mixture was left to stand at room temperature for 10 min, then centrifuged for 3 min at 18,000 g. Unbound alcian blue was determined from OD$_{600}$ of the supernatant, with reference to a standard curve prepared with alcian blue solutions ranging from 0 to 0.05% (w/v) 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 calcoflour white (23). Cells were cultured with or without CQ either overnight or for 3 h, then adjusted to 1 x 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 365nm laser. Calcofluor white was detected with a 440/40BP emission filter.

**Chloroquine uptake.** Chloroquine uptake by cells was estimated using a fluorescently-labelled chloroquine molecule, LynxTag-CQ™ Green (BioLynx Technologies). Aliquots (100 µl) of cells grown to OD$_{600}$~2.0 in YEPD broth were transferred to microfuge tubes. Chloroquine was added together with 2 µl of 1-mM LynxTag-CQ™ and, where specified, caffeine. Cells were incubated in the dark with shaking at 30ºC. Samples (10 µl) were removed at intervals and cells 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 with a 100X 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 deletant 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 on wild type growth, and this concentration was selected for screening. A total of 97 CQ-sensitive strains (growth ratio ≥1.45) were identified from the genome-wide 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 analyzed for significant differences compared with genome-wide distributions for *S. cerevisiae*. ‘Stress Response’ was the most highly over-represented functional category in the annotations of genes in the CQ sensitive dataset, owing to the sensitivity of deletion strains including sat4Δ/sat4Δ, slt2Δ/slt2Δ and bck1Δ/bck1Δ (Table 1). The evident requirement for *SLT2* and *BCK1* in normal quinine resistance yielded additional over-represented categories, including MAPKKK cascade and directional cell growth.

The CQ sensitivity of cell-wall integrity pathway mutants is bck1Δ-, slt2Δ- and drug-specific. 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 screen criteria were stringent to avoid false positives (6). Whereas the *bck1Δ/bck1Δ* and *slt2Δ/slt2Δ* mutants showed marked growth defects versus 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, *bck1Δ/bck1Δ* and *slt2Δ/slt2Δ* 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 it is a non-compensatable defect in the pathway (brought about only by BCK1 or SLT2 deletion) that 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 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 like 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 or CW on CQ resistance. The bck1Δ/bck1Δ and slt2Δ/slt2Δ 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 sub-inhibitory 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 wild type, bck1Δ/bck1Δ and slt2Δ/slt2Δ 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 sub-inhibitory concentrations of caffeine or CQ, then 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 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: 1. The mode of CQ action against yeast involves targeting of the cell wall, similar to CW and caffeine. 2. Cell wall 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 #1), first we 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 β-1,3 glucan content (21), which did not differ significantly between CQ-exposed and non-exposed 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 sub-inhibitory 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≤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 ($chs1\Delta, chs2\Delta, chs3\Delta$) 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 similar CQ resistance as 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 (#2) that cell wall integrity is required for normal CQ resistance. Therefore, we performed sonication assays similar to those shown in Fig. 3 but where the drug was 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 removing CQ. In contrast, the continued presence of CQ would be required for sonication sensitivity if the relevant effect was to enable CQ entry to the cell. In the case of caffeine as a positive control, viability was decreased by $\geq 80\%$ due to sonication whether 4 mM caffeine was retained (Fig. 3) or removed just prior to sonication (Fig. 5A) (sonication without any caffeine treatment had 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. 5B), whereas 4 mM CQ during sonication produces a $\sim 80\%$ 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 directly, uptake of the drug was examined with a fluorescent probe approach (33). LynxTag-CQ uptake was approximately two-fold and three-fold higher in the $bck1\Delta/bck1\Delta$ and $slt2\Delta/slt2\Delta$ mutants,
respectively, than in wild type *S. cerevisiae* (Fig. 6A). This was in keeping with these mutants’ CQ sensitivities. Similarly, other treatments used above to perturb the cell wall (caffeine treatment and sonication) and which sensitized cells to CQ were also associated with increased LynxTag-CQ uptake (Fig. 6B,C). The results indicated that CQ toxicity is greater in cell wall-perturbed cells due to increased CQ uptake.

**Synergistic activity of caspofungin and chloroquine.** The dependency of chloroquine resistance on yeast cell wall integrity suggested the possibility that chloroquine could have 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 sub-inhibitory concentrations of CQ (Fig. 7). For example, simultaneous exposure to 2 mM CQ decreased the minimal inhibitory concentration (MIC) for CSP by approximately three fold. In contrast, only additive effects were observed with sub-inhibitory concentrations of other quinolone-containing drugs (Table 2). The above analyses also inferred a marked drop in the MIC for CQ in the presence of CSP, where ~20 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 for CSP was decreased by the presence of CQ also 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 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 successfully used as a tool in this study to elucidate how fungi with perturbed cell walls are hyper-sensitized 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). Here, the CQ-hypersensitivity of the *bck1Δ/bck1Δ* and *slt2Δ/slt2Δ* yeast deletion mutants highlighted a role for the mitogen-activated protein kinase (MAPK) cell wall integrity pathway (25-27). This was specific to CQ resistance, versus 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). As quinacrine was the only drug tested other than CQ where there was a suggestion of sensitivity in the *bck1Δ/bck1Δ* and *slt2Δ/slt2Δ* mutants, the alkyl side chain may be one structural feature that helps 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 for 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 chitin content of CQ exposed cells, but chitin was not a target of CQ action as chitin synthase defective cells were not affected for CQ resistance. This contrasted with 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 alkalinisation of the host environment and iron deprivation has been proposed (12, 14, 15). Pathogens like *C. albicans* themselves can actively alkalinise their environments (42), an action that could exacerbate CQ action. Related mechanisms of CQ action are known in *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 of fungal infections is well documented (3). For example, cell wall perturbing agents can be used in conjunction with antifungals such as polyenes and triazoles that target the cell membrane, a strategy that has proven beneficial in critically ill patients (43). Drug combinations may reduce the risk of resistance, adverse effects and improve treatment duration. The use of one antifungal to increase penetration of another was exploited here with CQ and the cell wall-targeting echinocandin drug caspofungin (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 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, sub-inhibitory 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 for caspofungin was evident *in vitro* was 125 µM CQ. This concentration is higher than is typically encountered in the clinical setting. *In vivo* concentrations in the plasma 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 alkalinisation (12, 14, 15) could be expected to be markedly exacerbated in vivo. Thus, fungal pathogens would be likely to be more susceptible to caspofungin-dependent CQ uptake during in vivo infection. Accordingly, the synergistic activities 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. The findings could prove especially relevant to malaria patients with serious fungal infections (48, 49).

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REFERENCES


**TABLE 1** Over-representation of specific functional categories in the annotations of genes from the chloroquine-sensitive dataset

<table>
<thead>
<tr>
<th>Functional category</th>
<th>P value</th>
<th>Genes</th>
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<tbody>
<tr>
<td>Stress response</td>
<td>1.51 x10^{-3}</td>
<td><strong>YBR016W, SAT4, SLT2, BCK1</strong></td>
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<tr>
<td>Modification by phosphorylation, dephosphorylation, autophosphorylation</td>
<td>2.51 x10^{-3}</td>
<td><strong>SAT4, SLT2, BCK1, PTC5</strong></td>
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<tr>
<td>MAPKKK cascade</td>
<td>3.22 x10^{-3}</td>
<td><strong>SLT2, BCK1</strong></td>
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<tr>
<td>Directional cell growth (morphogenesis)</td>
<td>4.51 x10^{-3}</td>
<td><strong>SLT2, BCK1</strong></td>
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<tr>
<td>Vacuole or lysosome</td>
<td>8.41 x10^{-3}</td>
<td><strong>VPS41, TLG2</strong></td>
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</table>

*a* The dataset comprises the defective gene function of each deletion strain that scored chloroquine sensitive.

*b* All functional categories scoring $P \leq 0.01$ are shown.

*c* According to [http://funspec.med.utoronto.ca/](http://funspec.med.utoronto.ca/)
TABLE 2 Antifungal Etest assays of caspofungin and chloroquine drug combinations

<table>
<thead>
<tr>
<th>Organism</th>
<th>Drug</th>
<th>Concentration (mM)</th>
<th>Caspofungin MIC&lt;sup&gt;a&lt;/sup&gt; (µg ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Effect&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>C. albicans SC5314</td>
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<td></td>
<td>CQ&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup>MIC, Minimum inhibitory concentration. Results from triplicate determinations were identical, so errors are not shown.

<sup>b</sup>For definitions of synergy and additivity see the legend to Figure 7.

<sup>c</sup>When supplied alone, CQ was non-inhibitory at all the indicated concentrations. The MICs for CQ were: C. albicans, 15 mM; C. glabrata, 4 mM; A. fumigatus, 0.5 mM; S. cerevisiae, 20 mM.
FIG 1 Chloroquine sensitivity of strains defective for *BCK1* or *SLT2*. Exponential phase cultures of *S. cerevisiae* BY4743, and isogenic strains *bck1Δ/bck1Δ* and *slt2Δ/slt2Δ* were serially diluted and spotted to agar supplemented or not with drugs (CQ, chloroquine; AQ, amodiaquine; MQ, mefloquine; QCR, quinacrine; QN, quinine) at the indicated concentrations. Images were captured after incubation for 48 h at 30°C.

FIG 2 Rescue of chloroquine sensitivity by sorbitol, and synergistic action with caffeine. (A) Exponential phase cultures of *S. cerevisiae* BY4743 (WT), and isogenic strains *bck1Δ/bck1Δ* and *slt2Δ/slt2Δ* were serially diluted and spotted to agar supplemented or not with CQ, caffeine or calcofluor white (CW) at the indicated concentrations and in the absence (left panels) or presence (right) of 1 M sorbitol. (B) Strains were spotted to agar supplemented with the agents at concentrations that were just sub-inhibitory to the relevant strains(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. Percentage viability was calculated from colony forming unit 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). Mean ±SEM values are shown from three independent determinations.
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 then probed for chitin content with 1.1 μM CW (this concentration is non-inhibitory to BY4743). Mean values ±SEM are shown from three independent determinations. (B) Exponential phase cultures of *S. cerevisiae* YPH499 (wild type) and a chitin synthase-defective derivative strain YMS348s (*chs1Δ,chs2Δ,chs3Δ*) were serially diluted and spotted to agar supplemented or not with 22 μM CW or 25 mM CQ. Images were captured after incubation for 48 h at 30°C.

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 drug then sonicated for 1 min. Percentage viability was calculated from colony forming unit 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 from 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 strains *bck1Δ/bck1Δ* (●) and *slt2Δ/slt2Δ* (■) were incubated in the presence of 0.4 mM chloroquine spiked with 20 μM LynxTag-CQ™. Cellular LynxTag-CQ™ was determined at intervals with flow cytometry. (B) LynxTag-CQ™ 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™ uptake was determined in BY4743 cells during incubation with 4 mM CQ, before or after sonication for 1 min. All values are means ±SEM from three independent determinations.
Synergistic action of caspofungin and chloroquine against *Candida albicans*. (A) *C. albicans* SC5314 was spread plated to RPMI-2G agar and overlaid with an Etest caspofungin strip. Images of growth were captured after 48 h incubation at 37°C. (B) The MIC for caspofungin was determined from Etest analysis (A) in agar supplemented with different sub-inhibitory 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 control. Additivity (or indifference) was defined as a decrease of <3 (34, 50). When supplied alone, CQ was non-inhibitory at all the indicated concentrations.
The graphs show the viability retained after sonication for different concentrations of caffeine and chloroquine. The y-axis represents the percentage viability retained, and the x-axis represents the concentration in mM.

For caffeine, the viability remains high across all concentrations, with slight decreases at higher concentrations.

For chloroquine, there is a clear trend showing a decrease in viability as the concentration increases, with a significant drop at 4 mM.
Figure A: Bar graph showing the normalized Calcofluor white fluorescence levels with varying concentrations of Chloroquine (0, 10, 20, 30 mM). The fluorescence increases with higher concentrations of Chloroquine.

Figure B: Images comparing wild type and chs1,2,3Δ strains before and after treatment with Calcofluor white and Chloroquine. The wild type strain shows increased fluorescence under both conditions, while the chs1,2,3Δ strain shows decreased fluorescence, indicating a defect in chitin synthesis.