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Quinacrine Inhibits *Candida albicans* Growth and Filamentation at Neutral pH

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Candida albicans **is a common cause of catheter-related bloodstream infections (CR-BSI), in part due to its strong propensity to form biofilms. Drug repurposing is an approach that might identify agents that are able to overcome antifungal drug resistance within biofilms. Quinacrine (QNC) is clinically active against the eukaryotic protozoan parasites *Plasmodium* and *Giardia*. We sought to investigate the antifungal activity of QNC against *C. albicans* biofilms. *C. albicans* biofilms were incubated with QNC at serially increasing concentrations (4 to 2,048 μg/ml) and assessed using a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) assay in a static microplate model. Combinations of QNC and standard antifungals were assayed using biofilm checkerboard analyses. To define a mechanism of action, QNC was assessed for the inhibition of filamentation, effects on endocytosis, and pH-dependent activity. High-dose QNC was effective for the prevention and treatment of *C. albicans* biofilms *in vitro*. QNC with fluconazole had no interaction, while the combination of QNC and either caspofungin or amphotericin B demonstrated synergy. QNC was most active against planktonic growth at alkaline pH. QNC dramatically inhibited filamentation. QNC accumulated within vacuoles as expected and caused defects in endocytosis. A tetracycline-regulated *VMA3* mutant lacking vacuolar ATPase (V-ATPase) function demonstrated increased susceptibility to QNC. These experiments indicate that QNC is active against *C. albicans* growth in a pH-dependent manner. Although QNC activity is not biofilm specific, QNC is effective in the prevention and treatment of biofilms. QNC antibiofilm activity likely occurs via several independent mechanisms: vacuolar alkalinization, inhibition of endocytosis, and impaired filamentation. Further investigation of QNC for the treatment and prevention of biofilm-related *Candida* CR-BSI is warranted.**

The ability of *Candida albicans* to form biofilms is a key attribute that enhances its ability to cause opportunistic infections (1, 2). In sessile form, *C. albicans* undergoes pleiotropic phenotypic changes, leading to protection from host immune defenses and increased resistance to antifungal therapy (3–6). Moreover, biofilms serve as a protected nidus from which subsequent dissemination via the bloodstream may occur. A wide range of adjunctive systemic therapies have been investigated for use in combination with traditional antifungal therapy in order to improve efficacy against invasive or disseminated candidiasis (3, 7, 8).

Quinacrine (QNC), a water-soluble acridone derivative, was widely used for the prevention and treatment of malaria during World War II and remains available as a highly active therapeutic agent against giardiasis. The pharmacologic effects of QNC remain incompletely defined but include the inhibition of nucleic acid synthesis through DNA intercalation and antiprostaglandin and antiplatelet effects due to the inhibition of phospholipase A2 and antilipolytic activity, the inhibition of platelet aggregation, and accumulation within neutrophils (9–12).

QNC has activity against a number of protozoa, including *Plasmodium*, *Giardia*, and *Leishmania* species. QNC accumulates within the vacuole of the malarial parasite, where it is thought to inhibit hematin polymerization. Additionally, the antifungal activity of QNC has been demonstrated in a limited number of *in vitro* studies. High concentrations of QNC inhibit the growth of the yeast *Saccharomyces cerevisiae* (13, 14), and QNC, which normally accumulates in vacuoles, has long been used as a marker for the *S. cerevisiae* vacuole in cell and molecular biology (15). A study from 1975 showed that in proline-rich medium, QNC inhibited *C. albicans* mitochondrial synthesis, and QNC concentrations of >1 mM decreased filamentation (16). In *Cryptococcus neoformans*, QNC and, to a lesser degree, the closely related compound chloroquine, have been shown to exhibit anticryptococcal activity; chloroquine was shown to disrupt pH-dependent cellular processes after accumulation within the vacuoles (17, 18).

We therefore sought to determine whether QNC was effective for the prevention and treatment of *C. albicans* biofilms and to analyze the mechanistic effects of QNC, with a focus on filamentation and vacuolar function. We used a static microplate model of *C. albicans* biofilms to systematically determine the optimal concentration of QNC needed to (i) inhibit mature preformed *C. albicans* biofilms, (ii) prevent *C. albicans* biofilm formation, (iii) determine its activity when combined with standard antifungal drugs, and (iv) identify potential mechanisms of action of QNC antifungal activity, with the overall objective of defining the antifungal activity of QNC and its utility against *C. albicans* biofilms.

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MATERIALS AND METHODS

Strains and reagents. The clinically derived wild-type strain *C. albicans* SC5314 was used as a reference isolate for the detailed studies. The *C. albicans* clinical isolates ATCC 10231, ATCC 14053, and ATCC 24433 and the echinocandin-resistant clinical isolates 42379 and 53264 were also studied (4, 19). For biofilm formation, the strains were grown overnight at 30°C in yeast extract-peptone-dextrose (YPD) medium (Fisher Scientific). The harvested cells were washed twice and resuspended in phosphate-buffered saline (PBS) (Sigma-Aldrich). For the biofilm studies, a standardized cell suspension was made in RPMI 1640 (Mediatech) supplemented with L-glutamine (Gibco) and buffered with 165 mM morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich) to pH 7.0, to attain a cell density of 1×10^6 cells/ml. Mechanistic studies of the vacuole utilized strains impaired in vacuolar ATPase (V-ATPase) function that were previously studied by our group: (i) the *vph1*Δ mutant and its reintegrant, *vph1*Δ plus *VPH1*, the *stv1*Δ mutant and its reintegrant, *stv1*Δ plus *STV1*, and DAY185 as a positive control (20), and (ii) tetR-*VMA3*, a conditional mutant in which *VMA3* is under the control of a tetracycline-regulated promoter (21), and its positive control with THE1-CIp10, in which the *URA3* gene has been integrated into the genome (22). The *vph1*Δ and *stv1*Δ mutants are each deficient in one of the two isoforms of the V_oa subunit of V-ATPase, *VPH1* or *STV1*. Specifically, the *vph1*Δ mutant is deficient in V-ATPase at the vacuolar membrane, and the *stv1*Δ mutant is deficient in V-ATPase at the Golgi apparatus and prevacuolar compartments (23). In tetR-*VMA3*, treatment with 20 μg/ml doxycycline (DOX) abolishes all V-ATPase activity.

Biofilm formation and susceptibility assays. The antifungal activity of quinacrine dihydrochloride (QNC) (Fluka Biochemika) against preformed mature biofilms for the prevention of biofilm formation and pH dependence was assessed using an XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] (Sigma-Aldrich) reduction assay in a standard 96-well static microplate model, as described by Ramage and López-Ribot (24). In detail, 100 μl of the standardized cell suspension was added to designated wells of 96-well microtiter plates, and cell-free RPMI 1640 was added to designated negative-control wells; the plates were incubated at 37°C for 24 h to allow for biofilm formation. The mature biofilms were washed gently three times with PBS and incubated again for 24 h at 37°C with increasing concentrations (4 to 2,048 μg/ml in doubling serial dilutions in buffered RPMI 1640) of QNC. Drug-free buffered RPMI 1640 was added to the wells that were designated positive controls (i.e., no treatment). After QNC treatment, the biofilms were again washed gently with PBS. The XTT reduction assay was used to determine the metabolic activities of the biofilms (24). The microtiter plates were incubated at 37°C for 1.5 to 2 h to allow the optimal formation of formazan. The color intensity of XTT was measured at an optical density of 490 nm (OD_{490}) on a BioTek ELx808 microplate reader (BioTek Instruments). The antifungal activity of each QNC treatment was expressed as a percentage relative to the metabolic activity of the untreated biofilms. Each biofilm experiment was repeated three times independently, and the results presented are the mean values from all three experiments.

To test the pH dependence of QNC antifungal activity in the prevention of biofilm formation, we used the protocol described above, except that the cells were incubated in RPMI 1640 buffered to a specified pH; the acidic pH buffers comprised 50 mM succinic acid–50 mM Na_2PO_4, and the alkaline pH buffers comprised 50 mM morpholineethanesulfonic acid (MES) hydrate–50 mM MOPS.

To test for interactions between QNC and known antifungals against mature biofilms, we used a checkerboard assay (25). Twofold dilutions of amphotericin B (AMB), caspofungin (CAS), or fluconazole (FLC) were prepared according to CLSI guidelines (26). After the formation of biofilms, serially increasing concentrations of QNC and antifungal drugs were combined. After 24 h of coincubation, the XTT assay was used to determine biofilm metabolic activity.

Growth curves and growth at various pH levels. Growth was assessed in complete synthetic medium (CSM) by coincubating strains with serially increasing concentrations of QNC and measuring the OD_{600} at fixed intervals in an automated Synergy H1M microplate reader (BioTek Instruments). The growth curves were generated using Prism 6.0 (GraphPad Software, Inc.). Growth at pH 4.0 to 8.5 was also tested in liquid CSM: *C. albicans* SC5314 was tested for growth at various pH levels (4.0 to 8.5) using pH-buffered CSM with or without QNC (1,024 μg/ml). *C. albicans* SC5314, V-ATPase-impaired strains, and their controls were tested for growth at various pH levels (4.0 to 8.5) using pH-buffered CSM with agar with or without QNC (1,024 μg/ml), and with and without DOX for THE1-CIp10 and tetR-*VMA3*. The cells from the overnight cultures were washed and counted as previously described (19). PBS was inoculated with the cells from overnight cultures to a starting density of 10^8 cells/ml. Next, a total of six 5-fold dilutions (1.0×10^8, 2.0×10^7, 4.0×10^6, 8.0×10^5, 1.6×10^5, 3.2×10^4, 6.4×10^3, and 1.3×10^3 cells/ml) were completed in 96-well plates, and the cells were spotted onto agar plates using a multiblot replicator (VP Scientific) and incubated at 37°C for 48 h.

Filamentation assays. The solid media tested were YPD with 10% fetal calf serum (FCS) (Invitrogen), medium 199 supplemented with L-glutamine, and RPMI–L-glutamine. All filamentation media were prepared with or without 1,024 μg/ml QNC, and all filamentation media were prepared with 2% (wt/vol) agar. Three microliters of SC5314 cells from overnight cultures was spotted onto agar plates and incubated at 37°C for 120 h. Liquid RPMI 1640 with or without 1,024 μg/ml QNC was inoculated with the cells from the overnight cultures to a starting density of 5×10^6 cells/ml, grown at 37°C, and centrifuged at 200 rpm for 2 to 24 h. The cells were visualized via light microscopy at selected time points.

Vacuolar morphology and fluorescence imaging. FM4-64 is a lipophilic dye that stains membranes and is actively endocytosed to the vacuole; due to these properties, it has been used extensively as both a vacuolar membrane stain and an endocytic marker. To stain vacuoles with FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl)hexatrienyl) pyridiniumdibromide] (Life Technologies), the cells were first grown in YPD overnight. Next, the cells were washed in 1× PBS and then resuspended in fresh YPD with or without 1,024 μg/ml QNC and grown for 4 h. The cells were resuspended to an OD_{600} of 2 to 4 in YPD with 40 μM FM4-64, incubated for 15 min at 30°C, and then washed and resuspended in fresh YPD and incubated for 60 min at 30°C, with images taken every 15 min via microscopy using the Texas Red filter. A Zeiss Imager M1 system was used for capturing images using differential inference contrast (DIC) and fluorescence microscopy, and rendering was completed using the AxioVision 4.7 software (Zeiss). To quantify vacuolar morphology, images of ≥10 random fields were taken per condition, and the number of visible vacuolar vesicles in 100 cells per experiment was determined; next, the cells were grouped into one of three categories (1 to 2, 3 to 4, or ≥5 vacuoles/cell). The mean data from three independent experiments with their standard deviations are presented here, as previously shown in Baars et al. (27).

Statistical analyses. The metabolic activities of the prevention and treatment groups were compared to those of the controls using a one- or two-way analysis of variance (ANOVA) and Dunnett's multiple-comparison posttest. Differences were considered significant at a P value of <0.05. Statistical analyses were performed, and graphs were produced with GraphPad Prism 6.0 and Microsoft Excel.

Definitions. Antifungal activity was defined as a statistically significant reduction in the metabolic activity of mature *C. albicans* biofilms treated by QNC compared to the metabolic activity of the untreated biofilms. The MIC_{50} and MIC_{90} were defined as the concentration of QNC needed to reduce the metabolic activity of the biofilms of each strain by 50% and 90%, respectively. Fractional inhibitory concentrations (FICs) were calculated by the following formula: (MIC_{50} of drug A, tested in combination with another drug B)/(MIC_{50} of drug A alone) (28, 29). The FIC index (FICI) was defined as the FIC of drug A plus the FIC of drug B. In accordance with published guidelines (28), an FICI of ≤0.5 indicated

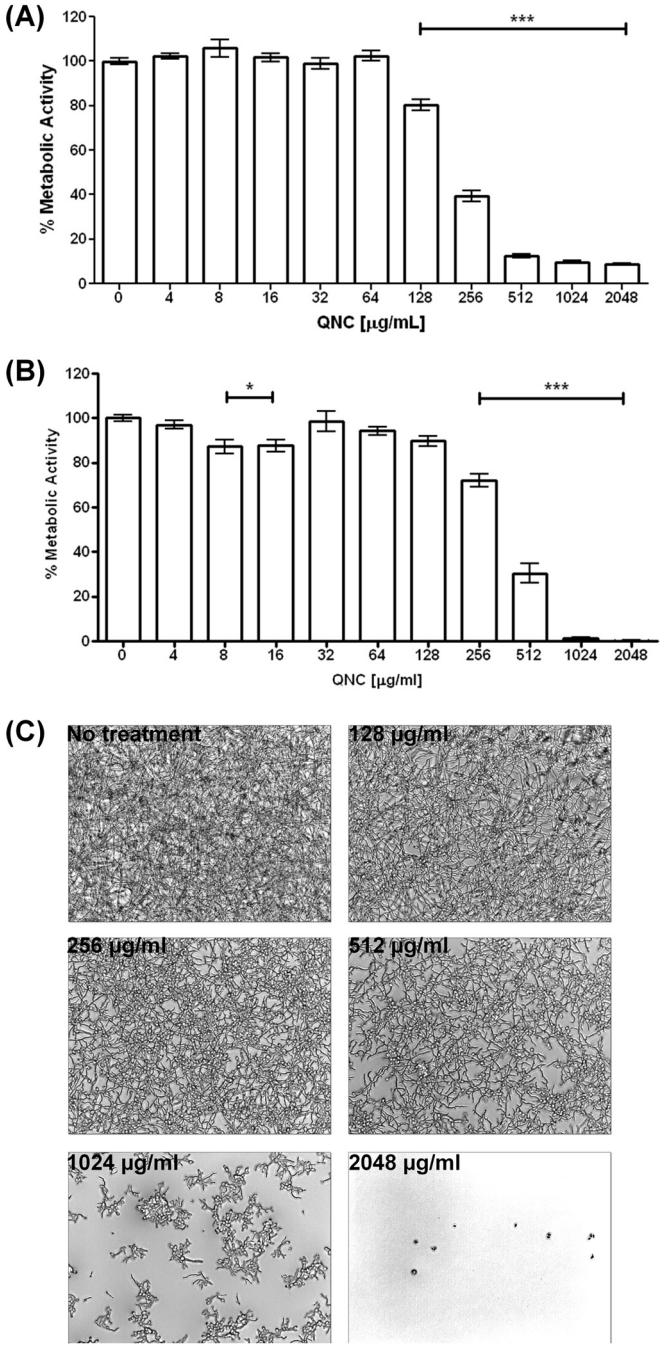

FIG 1 Effects of various quinacrine (QNC) concentrations on *C. albicans* SC5314 biofilm formation and mature biofilms. (A) *In vitro* effect of QNC against mature (preformed) *C. albicans* SC5314 biofilms were assayed in a 96-well static microplate biofilm model. The biofilms were challenged with buffered RPMI 1640 containing QNC at concentrations of 4 to 2,048 μg/ml for 24 h. MIC_{50}, 128 μg/ml; MIC_{80}, 512 μg/ml. (B) Effects of various concentrations of QNC against the formation of *C. albicans* SC5314 biofilms were assessed in a 96-well static microplate model. Cells were coincubated with RPMI 1640 containing QNC at concentrations of 4 to 2,048 μg/ml for 24 h. MIC_{50}, 256 μg/ml; MIC_{80}, 512 μg/ml. The bar graphs represent experiments performed three times independently (biological replicates), each time in quadruplicate (technical replicates). Metabolic activity was expressed as a percentage relative to the metabolic activity of the untreated biofilms. The statistical analysis was completed using ANOVA, and the significant differences ($P < 0.05$) in the reduction in biofilm metabolic activity with QNC compared to the no-treatment control (cells containing only medium and biofilms) are indicated by asterisks. Error bars indicate standard deviations. (C) Structural analyses of QNC activity against *C. albicans* SC5314 biofilm formation (prevention) were performed using standard light microscopy in a ×40 power field. The biofilms were formed as described above, and the biofilm morphology was assessed; representative images are shown. Visual differences in biofilm architecture are apparent at five different QNC concentrations (128, 256, 512, 1,024, and 2,048 μg/ml).

synergism, >0.5 to 4.0 indicated indifference, and >4.0 indicated antagonism.

RESULTS

Quinacrine demonstrates *in vitro* activity against *C. albicans* biofilms. The antifungal effect of 4 to 2,048 μg/ml QNC against mature *C. albicans* SC5314 biofilms was tested in a static microplate model (Fig. 1A). QNC at a concentration of >128 μg/ml caused a statistically significant reduction in *C. albicans* biofilm metabolic activity. The concentration of QNC needed to reduce biofilm (sessile) metabolic activity by 50% ($sMIC_{50}$) was 256 μg/ml, and the concentration needed to reduce biofilm (sessile) metabolic activity by 90% ($sMIC_{90}$) was 1,024 μg/ml. Next, we assayed the ability of QNC to prevent biofilm formation by coincubating SC5314 with serially increasing concentrations of QNC (Fig. 1B). QNC at a concentration of >256 μg/ml significantly decreased biofilm formation and, notably, at 1,024 μg/ml QNC biofilms were 98.5% inhibited. The $sMIC_{50}$ of QNC for biofilm prevention was 512 μg/ml, and the $sMIC_{90}$ was 1,024 μg/ml. A small but statistically significant difference was also seen at 8 and 16 μg/ml QNC. Thus, high-dose QNC was active for the prevention and treatment of *C. albicans* biofilms *in vitro*.

Light microscopy used to visualize gross biofilm structure indicated that the treatment of preformed biofilms with QNC resulted in a subtle reduction in overall biofilm density (data not shown). This modest difference is consistent with prior studies in our laboratory in which treatment with repurposed agents had little gross structural impact on mature biofilms (30, 31). In contrast, there was a dramatic reduction in biofilm density when coincubated with QNC in our model of biofilm prevention (Fig. 1C). Further, stunted hyphal cells were observed in biofilms coincubated with high doses of QNC (Fig. 1C). Next, the activity of QNC was tested against biofilms formed by *C. albicans* clinical isolates (see Fig. S1 in the supplemental material). The clinical isolates ATCC 10231, ATCC 14053, and ATCC 24433 were studied, as well as 42379 and 53264, two clinical isolates with mutations in the *fks1* gene conferring echinocandin resistance (4, 19). QNC demonstrated activity against both mature biofilms and biofilm formation for all five clinical isolates.

Next, the interaction of QNC with standard antifungal agents (amphotericin [AMB], caspofungin [CAS], or fluconazole [FLC]) was tested in biofilm checkerboard assays (Table 1). The combination of QNC with FLC against mature biofilms had no interaction (an "indifferent" effect). However, both AMB and CAS had synergy with QNC against mature biofilms. Thus, AMB and CAS act synergistically with QNC against mature preformed *C. albicans* biofilms.

Quinacrine activity is pH dependent. To test whether the effects of QNC on biofilms were due to growth inhibition, we tested the growth of QNC-treated planktonic cells in pH-buffered medium and on medium that had not been buffered to a specific pH (i.e., unbuffered medium). Planktonic growth with QNC was as-

TABLE 1 Biofilm checkerboard assays of various QNC concentrations in combination with various concentrations of antifungals

Expt[a]	Agent	$sMIC_{50}$ (mg/liter) of each agent: Alone	Combination	FICI (combination)[b]	Outcome[c]
QNC × AMB	AMB	0.25	0.03125	0.375	Synergism
	QNC	256	64		
QNC × CAS	CAS	0.125	0.03125	0.3125	Synergism
	QNC	64	4		
QNC × FLC	FLC	64	16	1.25	Indifference
	QNC	128	128		

[a] Concentrations of antibiotics used: amphotericin B (AMB), 0.0156 to 1 μg/ml; caspofungin (CAS), 0.0156 to 1 μg/ml; fluconazole (FLC), 2 to 128 μg/ml; QNC, 4 to 2,048 μg/ml. All were in 2-fold dilutions on *C. albicans* SC5314 mature preformed biofilms.

[b] FICI, fractional inhibitory concentration index.

[c] Synergy or indifference was demonstrated quantitatively in terms of the FIC and FICI definitions.

sessed for pH dependency in liquid CSM buffered to pH 4 to 8.5 with or without 1,024 μg/ml QNC for 30 h. The cells grew well at acidic pH, but no growth was detected in basic medium (Fig. 2A). Interestingly, in unbuffered medium, cells grown in the presence of QNC grew as well as the untreated cells (Fig. 2A). Additionally, serial dilutions of *C. albicans* SC5314 planktonic cells spotted onto unbuffered agar medium containing 16 to 2,048 μg/ml QNC showed no difference in colony growth (Fig. 2B). However, when *C. albicans* SC5314 cells were spotted onto solid medium buffered to pH 4.0 to 8.5 with or without QNC, no growth was seen on alkaline medium with QNC (Fig. 2C). Therefore, we conclude that QNC affects planktonic growth at alkaline pH but has no effect on growth on unbuffered medium.

We next tested QNC antibiofilm activity for pH dependency. QNC significantly decreased biofilm formation at pH 7.5 and 8.5, preventing 87.3% and 88.5% biofilm formation, respectively (Fig. 3). In contrast, QNC had only modest effects against *C. albicans* biofilm formation at more acidic pH (4.0 to 5.0) (Fig. 3). Therefore, the effects of QNC against biofilms are pH dependent and likely due to impaired growth at alkaline pH.

Quinacrine inhibits *C. albicans* filamentation. *C. albicans* biofilms are composed of complex communities of both hyphal and yeast cells surrounded by an extracellular matrix; the presence of hyphae is required for biofilm structural integrity. Because we observed less extensive filaments in the biofilms formed in the presence of high-dose QNC (Fig. 1C), and because we observed that cells grown on agar medium with QNC showed a smooth rather than wrinkly appearance (data not shown), suggesting a defect in filamentation, we next analyzed the effect of QNC on *C. albicans* hyphal formation. On unbuffered solid medium, filamentation was reduced by QNC in a dose-dependent manner under a variety of conditions (Fig. 4A). Hyphal formation in the presence of high-dose QNC was further studied via a 24-h time course in liquid RPMI. After 2 h of incubation with QNC, germ tubes had emerged, and QNC localized primarily to the vacuole (data not shown). By 6 h, filamentation was disrupted, with cells predominantly found as pseudohyphae (Fig. 4B). After 24 h, the cells remained in a pseudohyphal state. Notably, QNC fluorescence still localized to vacuoles but also displayed some cytosolic localization (Fig. 4B). Thus, QNC inhibits *C. albicans* filamentation while simultaneously localizing primarily to the vacuole.

Quinacrine inhibits active endocytosis and passive diffusion into the vacuole. Vacuolar morphology differs greatly between yeast and filamentous growth forms. In the yeast form, vacuoles are spherical, whereas hyphal vacuoles are enlarged and ellipsoid. Upon the induction of hyphal formation, vacuoles evaginate and segregate into pseudohyphal cells, where they later enlarge and elongate (32). Under hypha-inducing conditions, cells treated with high-dose QNC had vacuolar morphology resembling yeast or pseudohyphae rather than hyphae. After 4 h of QNC treatment, the vacuoles demonstrated evagination into pseudohyphae (data not shown), but by 6 h, the vacuoles in the QNC-treated cells remained spherical rather than ellipsoid (Fig. 4B). Due to these changes in the vacuole during morphogenic transitioning, we next sought to assay both the vacuolar morphology and functional aspects of vacuolar trafficking in the presence of high-dose QNC.

We utilized FM4-64, a lipophilic dye that is actively endo- and exocytosed. FM4-64 initially stains endocytic vesicle membranes and then accumulates at the vacuolar membrane before being actively exocytosed back to the plasma membrane (PM) (33). To assay for defective endocytosis, we treated yeast cells with QNC for 4 h, stained them with FM4-64, and imaged them at 15-min intervals. At 15 min, FM4-64 accumulated within discrete endocytic structures in the cell but did not reach the vacuolar membrane. At 30 min, FM4-64 was again visible at the PM, indicating active exocytosis. At 1 h, faint FM4-64 staining of the vacuolar membranes was visible, suggesting some degree of completed endocytosis to the vacuole (Fig. 5A). These data indicate an endocytic delay of approximately 45 min. Actively metabolizing cells contain multiple medium-sized vacuoles; in contrast, exposure to hypo-osmotic conditions causes the vacuole to enlarge, resulting in a single vacuole occupying a large volume of the cell (34). We observed that QNC treatment resulted in enlarged single vacuoles, compared to multiple smaller vacuoles observed in the controls. The quantification of this phenotype revealed that QNC treatment resulted in a significantly larger portion of cells with one to two vacuoles, whereas cells containing three to four vacuoles were more common in the controls (Fig. 5B).

Quinacrine affects growth of *C. albicans* V-ATPase mutants. The vacuolar-ATPase (V-ATPase) mediates the acidification of the vacuole, and strains of *S. cerevisiae* and *C. albicans* bearing genetic deletions of V-ATPase subunits are characterized by alkaline pH-conditional lethality (21, 35). Further, V-ATPase-deficient cells are viable on unbuffered medium but not on buffered medium at alkaline pH, mirroring the pH dependency of QNC (21). Functional V-ATPase allows for active transport of protons across the vacuolar membrane; accordingly, the proper assembly of V-ATPase is required for vacuolar acidification (21). We therefore tested whether the mechanism of action of QNC was related to V-ATPase activity using *C. albicans* strains previously studied in our laboratory with partial (*stv1*Δ and *vph1*Δ mutants) or total (tetR-*VMA3*) loss of V-ATPase function (20, 21). Both the *vph1*Δ and *stv1*Δ mutants were viable at all QNC concentrations, as were the DAY185 control and the isogenic *VPH1* and *STV1* reintegrant strains (Fig. 6A and B). Interestingly, upon repression of the *VMA3* gene with DOX, the tetR-*VMA3* mutant exhibited decreased growth with increasing QNC concentrations (Fig. 6C). Notably, minimal growth was seen at 1,024 μg/ml QNC, and no growth was seen at 2,048 μg/ml QNC (Fig. 6C). These results

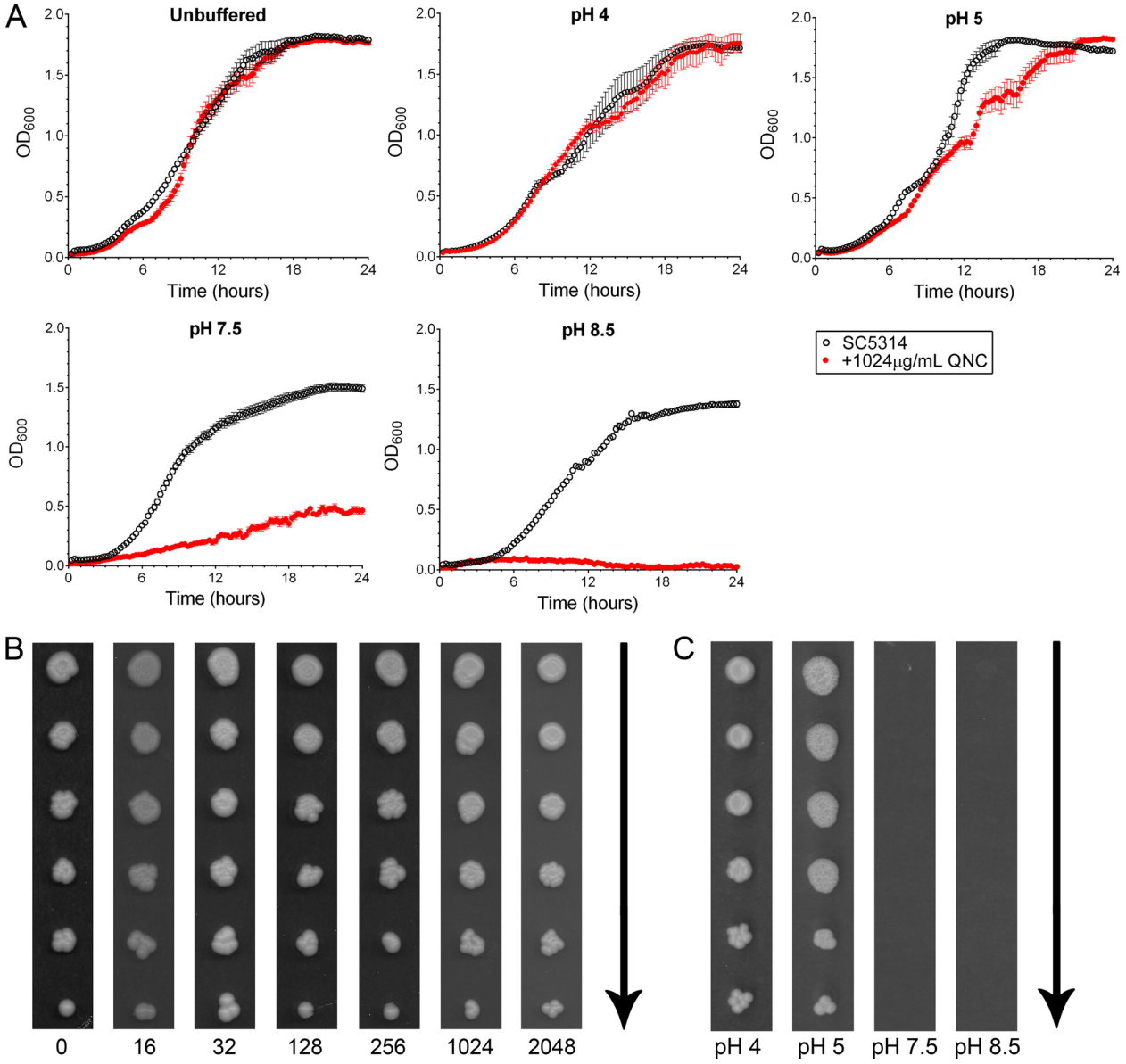

FIG 2 Effect of QNC on *C. albicans* SC5314 planktonic growth on unbuffered and pH-buffered medium. (A) Effect of QNC on growth in liquid medium at various pH levels. SC5314 cells from overnight cultures were diluted to an OD_{600} of 0.05 in CSM either without a pH buffer (unbuffered) or buffered to pH 4.0 to 8.5 as indicated, with or without 1,024 μg/ml QNC. The cells were grown at 30°C for 24 h, with OD_{600} readings taken at 15-min intervals. The growth of SC5314 with or without 1,024 μg/ml QNC, as indicated by the OD_{600} readings, is shown in a separate graph for each pH condition tested. The growth of SC5314 is indicated by open black circles, and the growth of SC5314 with 1,024 μg/ml QNC is indicated by closed red circles. (B) Effect of various QNC concentrations on growth on unbuffered CSM agar. Serial dilutions of overnight cultures of SC5314 were spotted onto agar medium containing various concentrations of QNC and incubated for 2 days at 30°C. The arrow to the right indicates decreasing cell densities (1.0×10^8, 2.0×10^7, 4.0×10^6, 8.0×10^5, 1.6×10^5, and 3.2×10^4 cells/ml, from left to right), and decreasing concentrations of QNC (0, 16, 32, 128, 256, 1,024, and 2,048 μg/ml) are noted on the bottom. (C) Effect of QNC on growth on solid medium at various pH levels. Serial dilutions of SC5314 overnight cultures were spotted onto solid CSM pH-buffered agar medium containing 1,024 μg/ml and incubated for 48 h at 30°C. The arrow to the right indicates decreasing cell densities (1.0×10^8, 2.0×10^7, 4.0×10^6, 8.0×10^5, 1.6×10^5, and 3.2×10^4 cells/ml, from left to right), and the pH of the medium is noted at the bottom.

suggest that QNC may act in a pH-dependent pathway that is parallel to that for the V-ATPase protein complex.

DISCUSSION

We systematically examined the activity of QNC against *C. albicans* biofilms, planktonic growth, and filamentation. Our findings indicate that high-dose QNC is effective for both the prevention and treatment of *C. albicans* biofilms. QNC at a concentration of ≥128 μg/ml caused a statistically significant decrease in the metabolic activity of preformed mature *C. albicans* SC5314 biofilms, and QNC at a concentration of ≥256 μg/ml caused a statistically significant decrease in biofilm formation. A concentration of 2,048 μg/ml QNC was required to completely prevent biofilm formation. Similar results were seen when QNC was utilized against clinical isolate biofilms. We also studied QNC in combination with traditional antifungal drugs using biofilm checker-

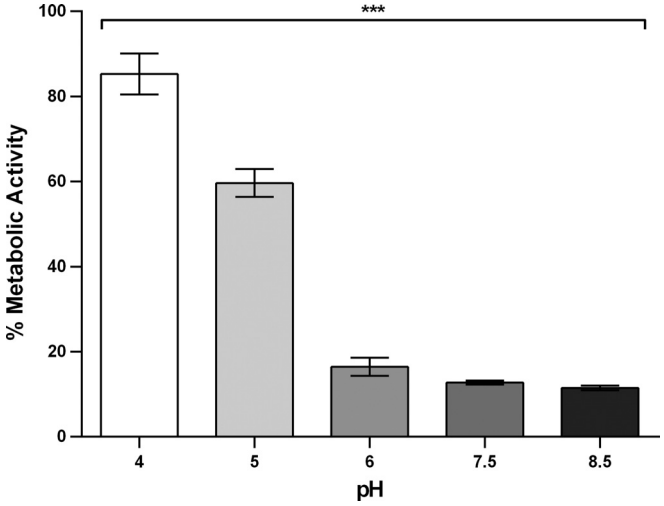

FIG 3 Effect of 1,024 μg/ml QNC on *C. albicans* SC5314 biofilm formation in pH-buffered RPMI 1640. The effects of various concentrations of QNC against the formation of *C. albicans* SC5314 biofilms were assessed in a 96-well static microplate model. The cells were coincubated with pH-buffered RPMI 1640 containing 1,024 μg/ml QNC for 24 h. The experiment was conducted three independent times with eight replicates per treatment, and a representative experiment is presented here. Error bars indicate standard deviations, and asterisks indicate statistical significance using one-way ANOVA ($P < 0.001$).

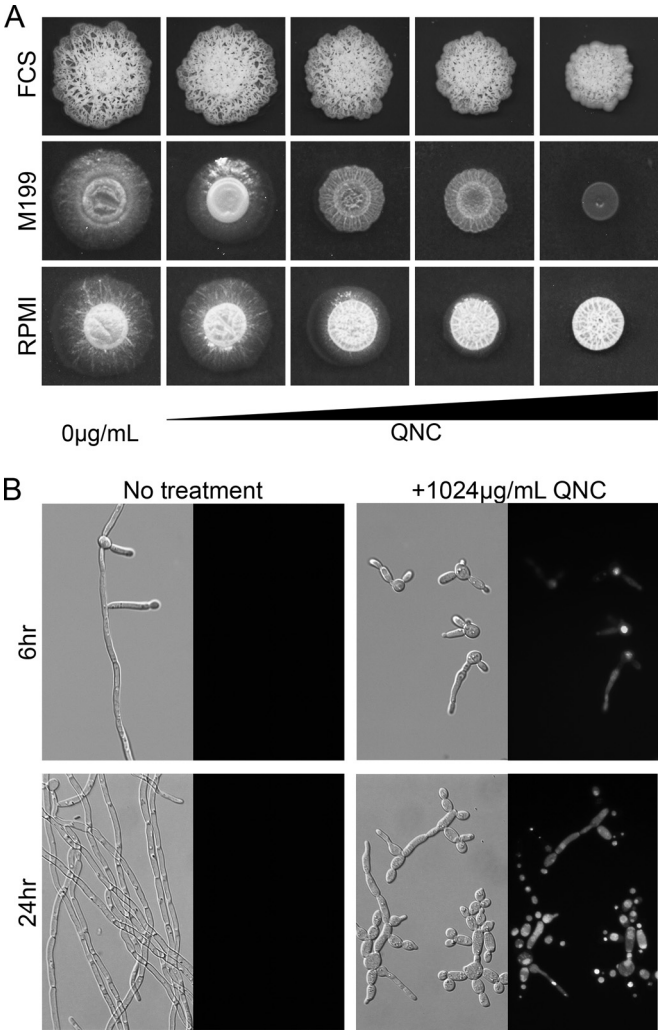

FIG 4 Effect of various QNC concentrations on *C. albicans* SC5314 filamentation on solid medium and 1,024 μg/ml QNC in liquid medium. (A) Growth of SC5314 colonies on YPD plus 10% fetal calf serum (FCS) solid medium, M199, and RPMI 1640, all with various concentrations of QNC (no treatment, 16, 128, 256, and 1,024 μg/ml, from left to right). (B) Filamentation of SC5314 in RPMI 1640 with and without 1,024 μg/ml QNC. Filamentation was assessed hourly by microscopy. DIC and QNC images at 6 (top) and 24 (bottom) h are shown.

board analyses and found that QNC and CAS, as well as QNC and AMB, display synergistic activity against *C. albicans* biofilms. Recently, high doses of AMB have been shown to cause vacuolar membrane fragmentation; this activity has been hypothesized to contribute to the fungicidal activity of AMB (36). Our data indicate that a sublethal dose of AMB (0.25 μg/ml) enhances the effectiveness of QNC against mature biofilms in a synergistic fashion. Further, we have shown that QNC localizes primarily to the vacuole, in accordance with many previous studies showing QNC accumulation in vacuoles. These data therefore suggest that the antifungal action of QNC occurs via a vacuole-specific mechanism. Quinacrine inhibition of vacuolar function is probably related to its basic properties. Under acidic conditions, the weakly basic QNC passively diffuses across membranes and accumulates in acidic compartments. There, QNC likely becomes protonated (i.e., by excess protons available under acidic environmental conditions, or that are available in acidic compartments even under alkaline conditions). This protonation is likely required for QNC fluorescence to occur (37). Thus, protonation of QNC in acidic vacuoles would interfere with normal vacuolar function by alkalinizing the vacuole. Under acidic environmental conditions, however, there would be enough excess protons available to quench quinacrine and thus avoid vacuolar alkalinization.

We also discovered a pH-dependent phenotype in which biofilms coincubated with QNC at acidic pH displayed only slightly reduced levels of both filamentation and biofilm formation, whereas biofilms coincubated with QNC at alkaline pH had minimal adherent cells, no filamentation, and no visible biofilm. The *C. albicans* response to host pH is well described and is critical for survival within the host, as well as for virulence (38). Consistent with this, *C. albicans* planktonic cells were unable to grow at alkaline pH in the presence of QNC in liquid or on solid medium. However, QNC-treated planktonic cells were able to grow as well as the untreated cells in unbuffered medium. Cells grown on unbuffered medium are able to modulate environmental pH (39); thus, these results suggest that *C. albicans* cells are able to negate the effects of QNC via the manipulation of extracellular pH. In this model, if extracellular pH cannot be modulated due to pH buffering, QNC affects both *C. albicans* growth and filamentation. Because the physiological pH of the mammalian host environment is under tight homeostatic regulation, QNC activity should be achievable *in vivo*. Further studies are required to test this hypothesis.

Filamentation is a major contributor to biofilm development and pathogenesis; we found that filamentation on solid medium was inhibited at a QNC concentration of >256 μg/ml in a dose-dependent manner. Additionally, we found that a QNC concentration of ≥1,024 μg/ml completely inhibited filamentation in liquid culture. Therefore, high-dose QNC inhibits *C. albicans* hypha formation. A previous study found that in the presence of

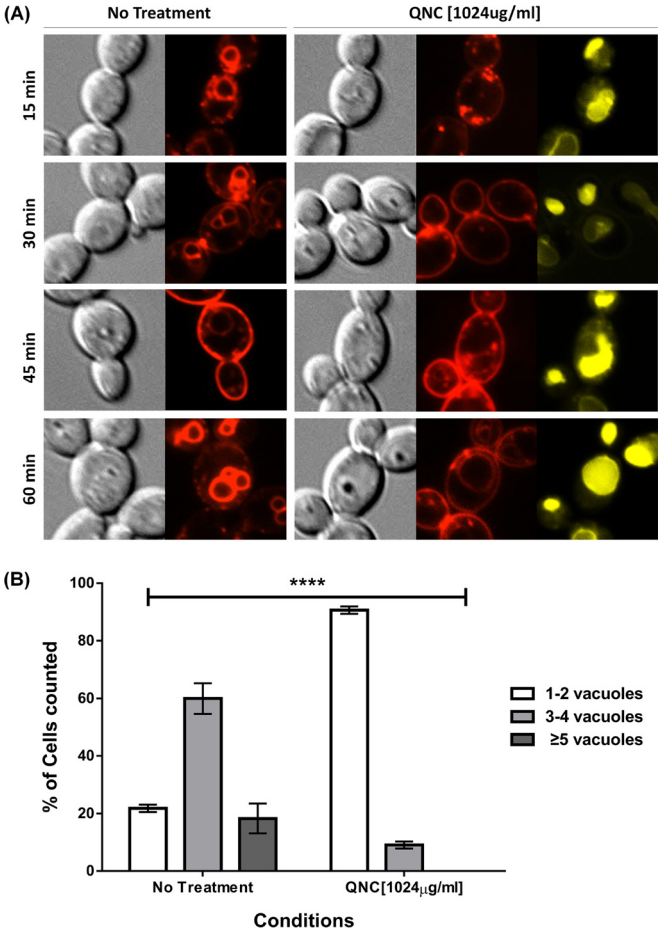

FIG 5 Effects of 1,024 μg/ml QNC on active endocytosis to the vacuole and vacuolar morphology. (A) SC5314 cells were incubated in the presence of 1,024 μg/ml QNC (yellow) for 4 h, followed by incubation with 40 μM FM4-64 (red); images were taken every 15 min to visualize active endocytosis from the plasma membrane (PM) via vesicles to the vacuole and back to the PM. (B) To quantify vacuolar morphology, photos of ≥10 random fields were taken per condition, and the number of visible vacuoles in 100 cells per experiment was determined. The cells were grouped into one of three categories (1 to 2, 3 to 4, or ≥5 vacuoles/cell) and then graphed as the percentage of cells counted per condition. The data from three independent experiments are presented, along with standard deviations. Asterisks indicate statistical significance using two-way ANOVA ($P < 0.0001$).

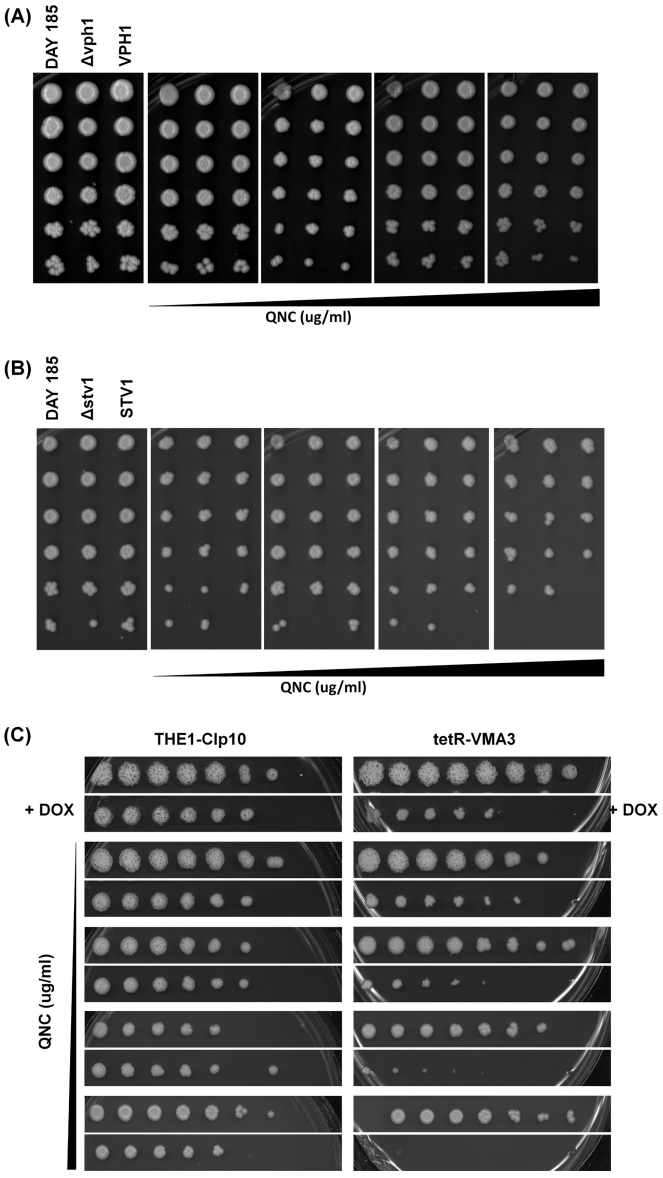

FIG 6 Effect of increasing QNC concentrations on the growth of *C. albicans* V-ATPase-deficient strains on solid medium. (A) Working with serial dilutions of DAY185, *vph1*Δ, and its reintegrant, *vph1*Δ plus *VPH1*, overnight cultures were spotted onto solid YPD agar medium containing QNC (no treatment, 256, 512, 1,024, and 2,048 μg/ml) and incubated for 2 days at 37°C. (B) Working with serial dilutions of DAY185, *stv1*Δ, and its reintegrant, *stv1*Δ plus *STV1*, overnight cultures were spotted onto solid YPD agar medium containing QNC (no treatment, 256, 512, 1,024, and 2,048 μg/ml) and incubated for 2 days at 37°C. (C) Working with serial dilution of THE1-CIp10 and tetR-*VMA3*, overnight cultures (after 24 h of preincubation with DOX) were spotted onto solid YPD agar medium, with and without DOX (indicated above as "+ DOX"), containing a range of QNC concentrations (no treatment, 256, 512, 1,024, and 2,048 μg/ml) and incubated for 2 days at 37°C.

proline, a filament inducer, a QNC concentration of >1 mM repressed hypha induction in *C. albicans* (16). Exploiting the ability of QNC to fluoresce, we found that QNC localized to vacuoles 2 h after induction of filamentation and by 24 h had dispersed throughout the cytosol. In *C. neoformans*, QNC uptake was diminished at low pH, suggesting that the weakly basic properties of QNC drive its localization to the vacuole (17, 40). We have shown that filamentation is impaired in the presence of QNC, in accordance with a previously published study showing that QNC decreased filamentation in the presence of proline (16). We have tested filamentation in the presence of QNC under a wide variety of other filamentation-inducing conditions, and we found that QNC treatment led to reduced filamentation under every condition tested. Therefore, QNC is a strong repressor of filamentation in *C. albicans*. A possible explanation is suggested by QNC localization to the cytosol after 24 h of incubation in filamentation-inducing liquid medium. At high extracellular pH, the pH gradient from the alkaline extracellular medium to the neutral cytosol and then to acidic organelles might drive QNC into acidified intracellular compartments. We hypothesize that QNC, a weak base, may partially alkalinize the acidic vacuole by taking up excess protons. After alkalinizing the vacuole, QNC would migrate to the now relatively acidic cytosol, disrupting pH-dependent processes

in *C. albicans*, including filamentation. However, at low extracellular pH, this gradient would not be present, preventing QNC accumulation in the internal organelles and thus allowing some degree of filamentation to occur. Mechanistic studies of pH-dependent regulation of filamentation are under way in our laboratories in order to further define these processes.

S. cerevisiae and *C. albicans* strains bearing genetic deletions of V-ATPase subunits develop a similar pH-dependent phenotype to QNC-treated cells, characterized by alkaline pH-conditional lethality (21, 35). Our results suggest that QNC inhibits acidification of the vacuole and acts in a manner that is similar or parallel to that of V-ATPase pumps. To further clarify this possibility, we treated *C. albicans* V-ATPase mutants with QNC. The V-ATPase complex consists of the V_1 and V_o subcomplexes, in which V_1 is responsible for ATP hydrolysis and V_o is responsible for proton transport into the vacuole (35). We previously studied *C. albicans* V-ATPase subunits V_oa, encoded by *VPH1* (the vacuolar V_oa subunit isoform) and *STV1* (the Golgi apparatus and endosomal V_oa subunit isoform), and V_oc, encoded by *VMA3* (20, 21). The loss of *VMA3* suppresses all V-ATPase activity and more severely inhibits filamentation than does the loss of *VPH1* or *STV1* in *C. albicans* (20), and this difference may implicate a mechanism whereby ATPase complexes at the vacuole and other organelles contribute to filamentation. These experiments demonstrate that similar to its effect on wild-type cells, QNC does not affect *vph1*Δ and *stv1*Δ planktonic growth. In contrast, tetR-*VMA3* demonstrates increased susceptibility to QNC, with 2,048 μg/ml QNC completely inhibiting planktonic growth. Because *vph1*Δ and *stv1*Δ mutants have partially impaired V-ATPase function in specific organelles but tetR-*VMA3* has fully impaired V-ATPase, a drug specifically targeting the V-ATPase would be expected to have a greater effect on growth of the *vph1*Δ and *stv1*Δ mutants than the strain with tetR-*VMA3*, in which V-ATPase is already fully impaired. However, we observed the opposite effect of QNC on these strains, suggesting that the antifungal activity of QNC does not work by disrupting V-ATPase complexes but rather by disrupting a pH-dependent pathway that functions in parallel with the V-ATPase complex.

Oral quinacrine has established efficacy for the systemic treatment of malaria and other parasitic diseases. In humans, QNC is highly and rapidly absorbed in the gastrointestinal tract after oral administration; of note, QNC is highly water soluble and has an extremely high volume of distribution. Thus, while serum concentrations of QNC are low, QNC achieves extremely high tissue concentrations, particularly in the spleen, pancreas, lungs, bone marrow, skeletal muscles, and especially in the liver (41). Concentrations in the liver may be 20,000 times that in the plasma (42). Due to accumulation in the tissue, QNC has a long half-life and is slowly excreted through urine and other bodily fluids (43). Thus, quinacrine may achieve clinically relevant concentrations in tissues, including the liver and spleen, which have direct relevance to disseminated candidiasis, particularly in combination with AMB or CAS, for which we demonstrated synergy *in vitro*. Common adverse drug reactions of QNC include gastrointestinal effects (e.g., nausea, diarrhea, and cramps) and dermatological effects, including rash, dermatitis, and yellowish skin discoloration (skin accumulation) (43, 44). Alternatively, high concentrations could be utilized as a local antifungal lock strategy in catheter-related bloodstream infections (7). Overall, these findings demonstrate that although QNC activity is not biofilm specific, QNC inhibits *C. albicans* growth, filamentation, and normal vacuolar function in a pH-dependent manner. Further investigation of the clinical utility and efficacy of QNC against invasive candidiasis and other invasive systemic mycoses is warranted.

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