Garcinia xanthochymus Benzophenones Promote Hyphal Apoptosis and Potentiate Activity of Fluconazole against *Candida albicans* Biofilms

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Xanthochymol and garcinol, isoprenylated benzophenones purified from *Garcinia xanthochymus* fruits, showed multiple activities against *Candida albicans* biofilms. Both compounds effectively prevented emergence of fungal germ tubes and were also cytostatic, with MICs of 1 to 3 μ M. The compounds therefore inhibited development of hyphae and subsequent biofilm maturation. Xanthochymol treatment of developing and mature biofilms induced cell death. In early biofilm development, killing had the characteristics of apoptosis, including externalization of phosphatidyl serine and DNA fragmentation, as evidenced by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) fluorescence. These activities resulted in failure of biofilm maturation and hyphal death in mature biofilms. In mature biofilms, xanthochymol and garcinol caused the death of biofilm hyphae, with 50% effective concentrations (EC_{50} s) of 30 to 50 μ M. Additionally, xanthochymol-mediated killing was complementary with fluconazole against mature biofilms, reducing the fluconazole EC_{50} from >1,024 μ g/ml to 13 μ g/ml. Therefore, xanthochymol has potential as an adjuvant for antifungal treatments as well as in studies of fungal apoptosis.

Disseminated *Candida albicans* infections represent a growing cause of morbidity and mortality, especially among immunocompromised individuals, surgical patients, and those with implanted medical devices. *Candida albicans* is a dimorphic fungus capable of existing as a commensal species on skin and mucus membranes of humans (1, 2). The fungus spends most of its life in the form of multicellular communities called biofilms, heterogeneous mixtures of yeast, hyphae, and pseudohyphal forms embedded in a complex extracellular matrix (3, 4). The plasticity of *C. albicans* growth is associated with the virulence of the organism. For instance, the hyphal form of the fungus is capable of invading and disrupting epithelial and endothelial cells, resulting in disseminated disease (5). Progression of disease is also a function of *C. albicans* dimorphism, since hyphae enable escape from macrophages (6). Fungal attachment and biofilm development contaminate implanted medical devices, necessitating removal of the devices to prevent dissemination (4). In biofilms, the fungus is refractory to removal by drug intervention or the host immune system. Indeed, numerous studies have associated biofilm formation with increasing drug resistance (7, 8). Multiple factors underlie the increased drug resistance observed in biofilms. These include upregulation of drug efflux pumps as well as biofilm architecture that results in differences in abundance of drug targets and metabolism within the biofilm community (4, 9). Further complicating the picture is the secretion of the extracellular matrix, which can function to impede the penetration of drugs throughout the biofilm (10). Concomitant with increasing drug resistance of *C. albicans* isolates, there is a dearth of drugs and drug targets for the treatment of systemic candidiasis (9). All current antifungal treatments target an extremely small part of the fungal genome (11). We are searching for new phytochemicals that are involved in protecting plants from fungal infection, realizing that they should have activity against biofilms, a common natural state of fungi.

We screened for compounds affecting biofilm integrity and

have identified xanthochymol and garcinol, stereoisomeric multi-isoprenylated benzophenones from the fruit of *Garcinia xanthochymus*, as potential antibiofilm agents (12). These compounds are present in high concentrations (up to 70 mg/g dry mass) (13). Xanthochymol and garcinol are catechols, compounds containing a 1,2-benzenediol moiety (Fig. 1) (12, 14, 15). Both garcinol and xanthochymol display biological activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (16). These compounds can also induce apoptosis in numerous human cancer cell lines, including leukemia and breast, prostate, colon, and pancreatic cancer cell lines (15–17). Garcinol inhibits histone acetyltransferase (HAT) p300 and pCAF at micromolar concentrations (18). In *C. albicans*, garcinol inhibits HAT Rtt109-catalyzed histone acetylation (19). Inhibition of microtubule disassembly into tubulin by xanthochymol has also been reported (20). Extracts from *Garcinia* species have antifungal activity against planktonic *C. albicans*, but only at the relatively high concentration of 50 mg/ml (21). However, biofilm-related properties of the purified benzophenones have not been reported previously. Analyses reported here ascribe an unusual activity to xanthochymol against *C. albicans* biofilms.

Received 6 April 2015 Returned for modification 28 April 2015

Accepted 12 July 2015

Accepted manuscript posted online 20 July 2015

Citation Jackson DN, Yang L, Wu S, Kennelly EJ, Lipke PN. 2015. *Garcinia xanthochymus* benzophenones promote hyphal apoptosis and potentiate activity of fluconazole against *Candida albicans* biofilms. Antimicrob Agents Chemother 59:6032–6038. doi:10.1128/AAC.00820-15.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00820-15>.

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FIG 1 Structures of xanthochymol and garcinol.

MATERIALS AND METHODS

***Candida albicans* strain and growth medium.** *Candida albicans* strain DAY185 (*ura3D::imm434/ura3D::imm434 HIS1::his1::hisG/his1::hisG ARG4::URA3::arg4::hisG/arg4::hisG*) forms robust biofilms (22, 23) and is resistant to the antifungal drug fluconazole; therefore, this strain was used in this study. *Candida albicans* was subcultured onto Sabouraud dextrose agar (per liter: peptone 10 g, dextrose 40 g, agar 15 g; pH of 5.6) and incubated at 30°C for 24 h. One colony was then added to yeast extract-peptone-dextrose (YPD; 1% yeast extract, 2% peptone, 2% dextrose) and incubated with shaking at 30°C and 170 rpm for 16 h, after which cells were harvested, washed in phosphate-buffered saline (PBS), and diluted to a standard inoculum of 1.0×10^6 cells/ml (24, 25).

***Garcinia* compounds.** Garcinol was obtained from Cayman Chemical. The purity of the compound was greater than 95%. Xanthochymol was isolated from a methanol extract from *Garcinia xanthochymus* fruits by reverse-phase column chromatography (15). Chemical structures were determined by one-dimensional and two-dimensional nuclear magnetic resonance and mass spectrometry (15). The purity of xanthochymol in all experiments was greater than 99%, as determined by high-pressure liquid chromatography (HPLC) (12).

Established biofilm assays. For biofilm assays in microtiter plates, 100 μ l of a standard inoculum of *C. albicans* cells (1.0×10^6 cells/ml) was added to the wells of a 96-well plate in triplicate, and the cells were allowed to adhere for 90 min at 37°C. After incubation, the plates were washed twice with PBS to remove nonattached cells. Then 100 μ l of RPMI 1640 with L-glutamine, without sodium bicarbonate (Gibco), buffered to pH 7.0 with 165 mM morpholinepropanesulfonic acid (MOPS) was added to each well, and the plates were incubated at 37°C for 24 h. Early-stage biofilms were prepared essentially as above, with the exception that the cells were induced to form biofilms for either 3 or 6 h prior to treatment.

Susceptibility testing of *Candida* biofilms. Fluconazole was obtained from Sigma. All drugs were dissolved in dimethyl sulfoxide (DMSO). After overnight growth of biofilms, plates were carefully washed to remove nonadherent cells, and then 100 μ l of 2-fold dilutions of xanthochymol and/or fluconazole, prepared according to Clinical and Laboratory Standards Institute guidelines, was added to the wells; then, the plates were returned to the incubator for a further 24 h.

Colorimetric readout based on XTT. Menadione and XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfonylphenyl)-2H-tetrazolium-5-carboxyanilide] were obtained from Sigma-Aldrich. After a drug challenge, the wells were washed twice with PBS, and then 100 μ l of XTT-menadione solution (4) was added to the wells, and plates incubated in the dark for 1 h at 37°C. The absorbance of wells at 490 nm was read using a microplate reader (Biotec/Synergy software).

Confocal microscopy. *Candida albicans* biofilms were treated with or without test compounds for various times, depending on the experimental design, after which the cells were prepared for microscopy by staining with the BacLight yeast viability kit, the contents of which include Syto-9 and propidium iodide (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Confocal images were obtained using an Olympus FluoView10i microscope and a filter set for Syto-9 and propidium iodide. Overlays included phase contrast images. All confocal images were processed using ImageJ.

Time lapse imaging. Bright-field images were obtained using an Olympus CKX41 cell culture microscope, attached to a Bioflux 200 automated microfluidic system for live cell imaging (Fluxion Biosciences, Inc.). Images were acquired using the Bioflux 200 camera driven by the Bioflux 200 software. All images were acquired using the 10 $\times$ objective with a zoom factor of 0.5.

Annexin V assays. Protoplasts of *C. albicans* early biofilms were obtained by incubation of the biofilm in potassium phosphate buffer (50 mM K_2HPO_4 , 5 mM EDTA, 50 mM dithiothreitol [DTT]), followed by treatment with 0.02 mg/ml zymolase (Sigma-Aldrich) for 10 min at 30°C. Protoplasts were washed and permeabilized on ice for 2 min in 0.1 M sodium citrate buffer containing 0.1% Triton X-100 (26, 27). The spheroplasts were stained with fluorescein isothiocyanate [FITC]-labeled annexin (Roche) according to the manufacturer's directions.

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling staining. Detection of DNA breakage, was accomplished using the *In Situ* Cell Death detection kit (Roche Life Sciences) according to the manufacturer's instructions. Briefly, biofilms were washed in PBS, followed by fixation in 4% paraformaldehyde for 1 h at 4°C. After fixation, biofilms were subjected to digestion with 0.02 mg/ml zymolase for 10 min at 30°C, followed by careful washing with PBS. Digested biofilms were permeabilized as indicated above and then incubated with 50 μ l of terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) reaction mixture for 1 h at 37°C.

Calculations. Biofilm viability was assayed by XTT reduction and calculated as follows:

$$\% \text{ viability} = 100 \times \frac{(\text{Abs}_{\text{well}} - \text{Abs}_{\text{background}})}{(\text{Abs}_{\text{untreated wells}} - \text{Abs}_{\text{background}})}$$

where background wells contained XTT solution only; untreated wells received cells only, with no added drug. The EC_{50} and 80% inhibitory concentration (IC_{80}) values were intercalated from the dose-response curves (4). The MIC was defined as the lowest concentration of drug capable of inhibiting growth, as measured by XTT assay, or the minimum concentration showing a significant increase in killing in the LIVE/DEAD assays.

RESULTS

Xanthochymol toxicity to *C. albicans* biofilms. Varalakshmi et al. reported antifungal activity of *Garcinia* extracts against *C. albicans* planktonic cells at 50 mg/ml (21). Consequently, we set out to determine whether purified garcinol and xanthochymol were active against biofilms at medically relevant concentrations. *Candida albicans* formed biofilms during incubation in RPMI medium at a pH of 7.0 and at 37°C for 24 h. These biofilms were then treated with xanthochymol or garcinol at 70 μ g/ml for a further 24 h. We performed LIVE/DEAD staining of the biofilm and quantified dead cells in confocal micrographs to ascertain if the compound was cytotoxic or cytostatic. In the presence of xanthochymol or garcinol, large propidium iodide-positive areas (red staining) were evident, characteristic of inviable cells with leaky membranes (Fig. 2A and B) (28). Close examination of treated biofilms showed that both compounds were effective at killing hyphae present in the mature biofilm, whereas the yeast form cells were Syto-9 positive (green), indicating they were viable. As a positive control, caspofungin (0.06 μ g/ml) showed killing of planktonic yeast phase cells but was less effective against a biofilm (Fig. 2B).

In contrast, when *C. albicans* DAY185 planktonic cells were grown in YPD in the presence or absence of either *Garcinia* benzophenone (70 μ g/ml), there was no significant effect on the growth or viability (Fig. 2C). We therefore conclude that both

FIG 2 Toxicity of *Garcinia* benzophenones against *C. albicans* biofilms. (A) Established biofilms were treated without or with xanthochymol or garcinol (70 µg/ml) for 24 h, after which the biofilms were stained with a LIVE/DEAD fluorescent stain and imaged by confocal microscopy. Green-fluorescent cells are alive, and red cells have compromised membranes and are not viable. (B) Caspofungin (0.06 µg/ml) is included as a control for cell death. It was more effective against planktonic cells (left) than biofilm (right). (C) Planktonic cells treated without or with xanthochymol for 24 h.

xanthochymol and garcinol were cytotoxic to *C. albicans* biofilms and specifically targeted the hyphal form of the fungus.

Early biofilm effects of *Garcinia* benzophenones. Biofilm formation involves several stages, including attachment, hyphal emergence, microcolony formation, and production of an extracellular matrix. The full process can take from 24 to 72 h (4). We examined the effects of xanthochymol on each phase of biofilm development. Xanthochymol did not significantly affect the attachment of the cells to glass slides coated with fibronectin (Fig. 3). However, after 3 h of treatment, it was apparent that the attached cells failed to form germ tubes and appeared to be locked in the yeast form of the growth. The fungus did not overcome the xanthochymol-induced block of the morphological switch even after 24 h under biofilm-inducing conditions (Fig. 3). Under these conditions, some yeast cells formed short linear chains, but there were few germ tubes, and the culture never developed true hyphae. In the presence of xanthochymol, the fungi also failed to form microcolonies (Fig. 3B, large arrows). Garcinol (70 µg/ml) also inhibited germ tube emergence after adhesion, but, unlike results with xanthochymol, the inhibition was transient, and germ tubes sometimes emerged after 6 h (data not shown). When germ tubes were allowed to emerge before treatment with xanthochymol, the germ tubes elongated and branched for several hours (see Fig. S1 in the supplemental material).

To determine whether the benzophenones could kill the biofilm cells during development or whether benzophenone activity was only cytostatic, we performed LIVE/DEAD staining on biofilms developed in the presence of the benzophenones (70 µg/ml). Consistent with our observations in Fig. 3, treatment of *C. albicans* biofilms after attachment but before germ tube emergence precluded hyphal development for 24 h (Fig. 4). Although there were clumps of yeast in the treated cultures, the biofilms were devoid of true hyphae in the xanthochymol-treated samples. The cells in these biofilms remained substrate attached, but many were killed by the drug. In the presence of garcinol, some germ tubes were observed, but the majority of cells in the culture were loosely attached or floating, indicating cell-cell aggregation but not cell-substratum attachment. Fewer cells were killed during garcinol treatment.

FIG 3 Effect of xanthochymol on early biofilm development. Time-lapse study of biofilm development in the presence of xanthochymol (35 µg/ml) (A) and without xanthochymol (B). Biofilm development times after adhesion are shown at the top of the figure. Continuous presence of xanthochymol in the culture inhibited the *C. albicans* development of hyphae and biofilm. Small arrows denote chains of yeast cells. Note the microcolonies apparent in the control culture at 3 and 9 h (large arrows).

FIG 4 Inhibition of biofilm maturation by *Garcinia* benzophenones. *C. albicans* cells were allowed to adhere, then nonadherent cells were removed, and biofilms were grown in an RPMI medium without or with garcinol or xanthochymol (70 µg/ml) for 24 h. LIVE/DEAD status of the biofilms was assayed by confocal microscopy. Fluorescence images (top) and overlays of fluorescence on phase contrast (bottom).

To assess the minimum concentrations of xanthochymol required for perturbation of early biofilms, we carried out dose-response analysis, adding xanthochymol to biofilms after 3 h of development. Xanthochymol concentrations in the low micromolar range (8.8 µg/ml, 13 µM) effectively induced cell death after 9 h of drug treatment (Fig. 5). Lower concentrations were cytostatic, with clear indications of hyphal inhibition at MICs near 1 µg/ml. Garcinol had similar activity.

Xanthochymol induced an apoptosis-like state in *C. albicans*. Investigations in mammalian model systems have established apoptosis and growth arrest as a result of xanthochymol intervention (16, 29). We therefore hypothesized that the observed cell death in our biofilms may be apoptotic in nature. Biofilms were developed for 3 h after adhesion, then treated without or with xanthochymol at 17.5 µg/ml for 3 h. To determine if features of apoptosis were present, we first verified the presence of DNA fragmentation with TUNEL staining, which was apparent in xanthochymol-treated cultures but not in the untreated samples (Fig. 6). A positive control of DNase-treated cells is shown in Fig. 6C. We also assayed for phosphatidyl serine (PS) externalization, as evidenced by binding of annexin V (Fig. 6D, green). The presence of annexin V staining indicated intact plasma membranes in *C. albicans* biofilms after the 3 h of xanthochymol treatment, indicating early apoptosis. Propidium iodide (red) counterstaining showed some yeast and hyphae with disrupted membranes and necrotic cell death as well (Fig. 6D). Thus, xanthochymol effects on early biofilms included induction of both apoptotic-like (Fig. 6) and necrotic-like cell death (Fig. 6 and 7), induction of cytostasis, and some cytotoxicity in yeast phase cells, depending on the xanthochymol dose and duration of exposure (Fig. 3 and 5).

Xanthochymol activity against established biofilms. To determine the activities of xanthochymol against mature biofilms, we treated mature biofilms with xanthochymol for 24 h and then stained for cell viability. There was massive death of hyphae at concentrations of 35 µg/ml and greater (see Fig. S2 in the supplemental material). Incidentally, xanthochymol treatment did not affect XTT reduction of the biofilm, perhaps because mitochondria

FIG 5 Dose response for xanthochymol effects on 3-h biofilms. *C. albicans* cells were allowed to adhere to fibronectin-coated coverslips for 1 h, and non-adherent cells were removed. Coverslips were then incubated at 37°C in RPMI medium for 3 h prior to addition of xanthochymol at the indicated concentrations. Developing biofilms were returned to the incubator for 9 h, after which the biofilms were washed and LIVE/DEAD stained.

drial function is severely downregulated in the biofilm state (see Fig. S3 in the supplemental material) (30).

Xanthochymol enhancement of fluconazole activity. Given that *C. albicans* within a biofilm can be up to 1,000-fold more resistant than planktonic yeast to fluconazole (31, 32), and given the specificity of xanthochymol for hyphae, we tested whether the two compounds might have complementary antifungal activities. We tested the combination of fluconazole and xanthochymol at 3 h and 24 h of biofilm development. In early biofilms, fluconazole at 32 µg/ml reduced biofilm activity by 20% (Fig. 7). Xanthochymol at 4 µg/ml reduced biofilm activity 65%. The effect of adding both drugs together was additive, resulting in a significant reduction in biofilm viability.

Established *C. albicans* DAY185 biofilms are markedly resistant to fluconazole. The 24-h biofilms showed only a 15% reduction in viability at fluconazole concentrations as high as 1,024 µg/ml by XTT assay (data not shown). We therefore tested xanthochymol and fluconazole in combination. After 24 h of growth, biofilms were treated with fluconazole, xanthochymol, or both drugs together. Following 24 h of exposure to the compounds, the biofilms were assayed by microscopy. As a single agent, fluconazole had minimal effect, whereas xanthochymol effectively killed hyphae (Fig. 8). The combination of fluconazole with xanthochymol killed most of the cells in the biofilm. Furthermore, the confocal micrographs showed the death of almost all hyphae, and many of the yeasts costained with both dyes; the nonred cells were markedly yellow compared to those in the control biofilm or the biofilms treated with fluconazole or xanthochymol alone. The yellow color is consistent with the remaining biofilm cells being in the process of dying. Double dilution assays showed that xanthochymol at 50 µg/ml reduced the fluconazole IC₅₀ to 13 µg/ml. Therefore, the drug combination was more effective than either drug alone and was toxic to all of the biofilm cells.

DISCUSSION

Xanthochymol and garcinol, isomeric polyisoprenylated benzophenones from *Garcinia xanthochymus* fruit, showed multiple activities against *C. albicans* biofilms. Several of these activities are novel, and they may enable use of these compounds in molecular studies of fungal development and may have potential for antibiofilm treatments. In temporal order, these activities included in-

FIG 6 Markers of apoptosis in xanthochymol-treated biofilms. The 3-h *C. albicans* biofilms were treated with or without xanthochymol (17.5 µg/ml) in RPMI medium for 3 h, then stained as indicated, and presented as fluorescent images (left) and fluorescent-phase overlays (right). (A) TUNEL-stained control (untreated) biofilm. (B) TUNEL-stained biofilm treated with xanthochymol. (C) TUNEL-stained biofilm treated with DNase I (3 U/ml). (D) Biofilm treated with xanthochymol and stained with annexin V (green) and propidium iodide (red). Bars, 33 µm (A and C) and 20 µm (B and D).

inhibition of hyphal emergence, inhibition of microcolony formation, proapoptotic activity early in biofilm formation, hyphal cytotoxicity in mature biofilms, and complementarity to the activity of fluconazole.

The ability of these compounds to delay the emergence of germ tubes under inducing conditions is an important outcome (Fig. 3, 4, and 5), because the yeast-hyphal transition is strongly linked to the establishment of *C. albicans* infection and cell and tissue dam-

FIG 7 A combination of fluconazole and xanthochymol decreases the viability of early biofilms. The 3-h biofilms were treated with xanthochymol (4 µg/ml) and/or fluconazole (32 µg/ml) for 6 h prior to XTT assay.

age (33). Once the germination program was initiated, xanthochymol did not initially prevent growth or branching of the hyphae (see Fig. S1 in the supplemental material). However, the compounds were cytotoxic to the developed hyphae over the course of 9 to 12 h (Fig. 4).

In vitro growth of *C. albicans* hyphae proceeds in two phases: in the first several hours, germ tubes are produced; then, in the second, they are extended to form true hyphae, which must be maintained long term (34). This process is under tight transcriptional regulation and is also dependent on chromatin remodeling (35, 36). A transcription complex between Tup1 and Nrg1 represses the initiation of transcription of hypha-specific genes. Induction signals transmitted through the cyclic AMP-protein kinase A (PKA) pathway result in loss of Nrg1 repression, followed by exchange of chromosomal remodeling enzymes, with the histone acetylase NuA4 dissociating and the histone deacetylase complex (HDAC) component Hda1 newly binding to the promoter region. Hda1 prevents rebinding of Nrg1 to hyphal promoters, probably by chromatin remodeling (37). The inhibitory effects of gancinol and xanthochymol on germ tube emergence imply that the compounds interfere with this program (38). It may be relevant that xanthochymol was effective at concentrations as low as 4 to 8 µg/ml (Fig. 7), the same concentration range at which gancinol inhibits histone acetylase activity in mammalian cells and in *C. albicans* NuA4 (17, 19).

Fungistatic and fungicidal activity. These results demonstrated the fungistatic and fungicidal activity in early biofilms after hyphal emergence, with an MIC of 1 µg/ml (1.6 µM) and a fungicidal IC₈₀ of 8.8 µg/ml (13 µM) (Fig. 5). Cell death resulting from xanthochymol treatment of *C. albicans* appeared to be similar to its caspase-dependent apoptotic effects in human cancer cells (15, 16, 29, 39). Xanthochymol-treated biofilms showed externalization of PS and DNA cleavage, as evidenced by TUNEL fluorescence (Fig. 6). The presence of TUNEL-positive cells in our assays speaks to the involvement of a caspase-like mechanism similar to that mediated by the *C. albicans* metacaspase Mca1 in re-

FIG 8 Treatment of *C. albicans* biofilms with fluconazole and xanthochymol. LIVE/DEAD-stained images of 24-h biofilms grown for an additional 24 h in the absence or presence of fluconazole (128 μ g/ml) and/or xanthochymol (100 μ g/ml).

sponse to oxidative stress (40–42). This metacaspase-dependent system is also activated in response to treatment with resveratrol, curcumin (43), propolis (44), amphotericin B (40), and caspofungin (26). Our results suggest that the *Garcinia* benzophenones in this study have the characteristics of metacaspase-dependent activators of apoptosis in *C. albicans* biofilms.

Interaction of fluconazole and xanthochymol. In this study, there was complementarity in the activities of xanthochymol and fluconazole. Fluconazole inhibits production of hyphae and hypha-specific wall proteins (18, 45). Our data show similarity in that xanthochymol-inhibited hyphal development (Fig. 3 and 4, and see Fig. S1 in the supplemental material). Therefore, the compounds may reinforce each other's activities. In contrast, caspofungin increases the expression of hypha-specific proteins (26, 46, 47), an antagonistic result. Therefore, it is not surprising that our assays have shown antagonism in efficacy assays of xanthochymol with caspofungin (data not shown).

In summary, xanthochymol and garcinol showed multiple activities against *Candida albicans*. Concentrations in the low micromolar range inhibited germ tube emergence and development of true hyphae. Intervention against early biofilms resulted in cytostatic and apoptosis-like responses. These activities are consistent with a possible function as an inhibitor of the fungal dimorphic switch. In mature biofilms, the compounds induced hypha-specific death, an action that could lead to the disruption and removal of the biofilm (Fig. 2, 4, 6, and 8; see also Fig. S2 and S3 in the supplemental material). Such activity should ameliorate hypha-induced cell and tissue damage in candidiasis (48–51). Furthermore, xanthochymol potentiated the activity of fluconazole. Therefore, these studies highlight the possible use of xanthochymol as well as garcinol in apoptosis studies and antifungal drug discovery.

ACKNOWLEDGMENTS

We thank Tresha Dorce and Judy Wright for consistent and high-quality support.

This work was supported by Public Health Service grant R01 GM098616.

REFERENCES

- Odds FC. 1985. Morphogenesis in *Candida albicans*. *Crit Rev Microbiol* 12:45–93. <http://dx.doi.org/10.3109/10408418509104425>.
- Sudbery P, Gow N, Berman J. 2004. The distinct morphogenic states of *Candida albicans*. *Trends Microbiol* 12:317–324. <http://dx.doi.org/10.1016/j.tim.2004.05.008>.
- Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. 1995. Microbial biofilms. *Annu Rev Microbiol* 49:711–745. <http://dx.doi.org/10.1146/annurev.mi.49.100195.003431>.
- Chandra J, Mukherjee PK, Ghannoum MA. 2008. In vitro growth and analysis of *Candida* biofilms. *Nat Protoc* 3:1909–1924. <http://dx.doi.org/10.1038/nprot.2008.192>.
- Gow NA, Brown AJ, Odds FC. 2002. Fungal morphogenesis and host invasion. *Curr Opin Microbiol* 5:366–371. [http://dx.doi.org/10.1016/S1369-5274\(02\)00338-7](http://dx.doi.org/10.1016/S1369-5274(02)00338-7).
- Wachtler B, Wilson D, Haedicke K, Dalle F, Hube B. 2011. From attachment to damage: defined genes of *Candida albicans* mediate adhesion, invasion and damage during interaction with oral epithelial cells. *PLoS One* 6:e17046. <http://dx.doi.org/10.1371/journal.pone.0017046>.
- Mukherjee PK, Chandra J. 2004. *Candida* biofilm resistance. *Drug Resist Updat* 7:301–309. <http://dx.doi.org/10.1016/j.drug.2004.09.002>.
- Nett JE. 2014. Future directions for anti-biofilm therapeutics targeting *Candida*. *Expert Rev Anti Infect Ther* 12:375–382. <http://dx.doi.org/10.1586/14787210.2014.885838>.
- Cowen LE, Sanglard D, Howard SJ, Rogers PD, Perlin DS. 2014. Mechanisms of antifungal drug resistance. *Cold Spring Harb Perspect Med* 5:pii=a019752. <http://dx.doi.org/10.1101/cshperspect.a019752>.
- Zarnowski R, Westler WM, Lacmabouh GA, Marita JM, Bothe JR, Bernhardt J, Lounes-Hadj Sahraoui A, Fontaine J, Sanchez H, Hatfield RD, Ntambi JM, Nett JE, Mitchell AP, Andes DR. 2014. Novel entries in a fungal biofilm matrix encyclopedia. *mBio* 5:e01333-14. <http://dx.doi.org/10.1128/mBio.01333-14>.
- Calderone R, Sun N, Gay-Andrieu F, Groutas W, Weerawarna P, Prasad S, Alex D, Li D. 2014. Antifungal drug discovery: the process and outcomes. *Future Microbiol* 9:791–805. <http://dx.doi.org/10.2217/fmb.14.32>.
- Acuña UM, Dastmalchi K, Basile MJ, Kennelly EJ. 2012. Quantitative high-performance liquid chromatography photodiode array (HPLC-PDA) analysis of benzophenones and biflavonoids in eight *Garcinia* species. *J Food Compos Anal* 25:215–220. <http://dx.doi.org/10.1016/j.jfca.2011.10.006>.
- Zhang LJ, Chiou CT, Cheng JJ, Huang HC, Kuo LM, Liao CC, Bastow KF, Lee KH, Kuo YH. 2010. Cytotoxic polyisoprenyl benzophenonoids from *Garcinia subelliptica*. *J Nat Prod* 73:557–562. <http://dx.doi.org/10.1021/np900620y>.
- Acuña UM, Figueroa M, Kavalier A, Jancovski N, Basile MJ, Kennelly EJ. 2010. Benzophenones and biflavonoids from *Rhedia edulis*. *J Nat Prod* 73:1775–1779. <http://dx.doi.org/10.1021/np100322d>.
- Baggett S, Protiva P, Mazzola EP, Yang H, Ressler ET, Basile MJ, Weinstein IB, Kennelly EJ. 2005. Bioactive benzophenones from *Garcinia xanthochymus* fruits. *J Nat Prod* 68:354–360. <http://dx.doi.org/10.1021/np0497595>.
- Matsumoto K, Akao Y, Kobayashi E, Ito T, Ohguchi K, Tanaka T, Iinuma M, Nozawa Y. 2003. Cytotoxic benzophenone derivatives from *Garcinia* species display a strong apoptosis-inducing effect against human leukemia cell lines. *Biol Pharm Bull* 26:569–571. <http://dx.doi.org/10.1248/bpb.26.569>.
- Balasubramanyam K, Altaf M, Varier RA, Swaminathan V, Ravindran A, Sadhale PP, Kundu TK. 2004. Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. *J Biol Chem* 279:33716–33726. <http://dx.doi.org/10.1074/jbc.M402839200>.
- Simonetti G, Passariello C, Rotili D, Mai A, Garaci E, Palamara AT. 2007. Histone deacetylase inhibitors may reduce pathogenicity and virulence in *Candida albicans*. *FEMS Yeast Res* 7:1371–1380. <http://dx.doi.org/10.1111/j.1567-1364.2007.00276.x>.
- Dahlin JL, Sinville R, Solberg J, Zhou H, Han J, Francis S, Strasser JM, John K, Hook DJ, Walters MA, Zhang Z. 2013. A cell-free fluorometric high-throughput screen for inhibitors of Rtt109-catalyzed histone acetylation. *PLoS One* 8:e78877. <http://dx.doi.org/10.1371/journal.pone.0078877>.
- Roux D, Hadi HA, Thoret S, Guenard D, Thoison O, Pais M, Sevenet

- T. 2000. Structure-activity relationship of polyisoprenyl benzophenones from *Garcinia pyrifera* on the tubulin/microtubule system. *J Nat Prod* 63:1070–1076. <http://dx.doi.org/10.1021/np0000872>.
21. Varalakshmi KN, Sangeetha CG, Shabeena AN, Sunitha SR, Vapika J. 2010. Antimicrobial and cytotoxic effects of *Garcinia indica* fruit rind extract. *American-Eurasian J Agric Environ Sci* 7:652–656.
22. Norice CT, Smith FJ, Jr, Solis N, Filler SG, Mitchell AP. 2007. Requirement for *Candida albicans* Sun41 in biofilm formation and virulence. *Eukaryot Cell* 6:2046–2055. <http://dx.doi.org/10.1128/EC.00314-07>.
23. Yang H, Figueroa M, To S, Baggett S, Jiang B, Basile MJ, Weinstein IB, Kennelly EJ. 2010. Benzophenones and biflavonoids from *Garcinia livingstonei* fruits. *J Agric Food Chem* 58:4749–4755. <http://dx.doi.org/10.1021/jf9046094>.
24. Pierce CG, Uppuluri P, Tummala S, Lopez-Ribot JL. 2010. A 96 well microtiter plate-based method for monitoring formation and antifungal susceptibility testing of *Candida albicans* biofilms. *J Vis Exp* 44:pii=2287. <http://dx.doi.org/10.3791/2287>.
25. Pierce CG, Saville SP, Lopez-Ribot JL. 2014. High-content phenotypic screenings to identify inhibitors of *Candida albicans* biofilm formation and filamentation. *Pathog Dis* 70:423–431. <http://dx.doi.org/10.1111/2049-632X.12161>.
26. Hao B, Cheng S, Clancy CJ, Nguyen MH. 2013. Caspofungin kills *Candida albicans* by causing both cellular apoptosis and necrosis. *Antimicrob Agents Chemother* 57:326–332. <http://dx.doi.org/10.1128/AAC.01366-12>.
27. Rieger AM, Hall BE, Luong LT, Schang LM, Barreda DR. 2010. Conventional apoptosis assays using propidium iodide generate a significant number of false positives that prevent accurate assessment of cell death. *J Immunol Methods* 358:81–92. <http://dx.doi.org/10.1016/j.jim.2010.03.019>.
28. Jin Y, Zhang T, Samaranayake YH, Fang HH, Yip HK, Samaranayake LP. 2005. The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in *Candida albicans* biofilms. *Mycopathologia* 159:353–360. <http://dx.doi.org/10.1007/s11046-004-6987-7>.
29. Protiva P, Hopkins ME, Baggett S, Yang H, Lipkin M, Holt PR, Kennelly EJ, Bernard WI. 2008. Growth inhibition of colon cancer cells by polyisoprenylated benzophenones is associated with induction of the endoplasmic reticulum response. *Int J Cancer* 123:687–694. <http://dx.doi.org/10.1002/ijc.23515>.
30. Traven A, Janicke A, Harrison P, Swaminathan A, Seemann T, Beilharz TH. 2012. Transcriptional profiling of a yeast colony provides new insight into the heterogeneity of multicellular fungal communities. *PLoS One* 7:e46243. <http://dx.doi.org/10.1371/journal.pone.0046243>.
31. Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. 2003. Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect Immun* 71:4333–4340.
32. Lamfon H, Porter SR, McCullough M, Pratten J. 2004. Susceptibility of *Candida albicans* biofilms grown in a constant depth film fermentor to chlorhexidine, fluconazole and miconazole: a longitudinal study. *J Antimicrob Chemother* 53:383–385. <http://dx.doi.org/10.1093/jac/dkh071>.
33. Nobile CJ, Nett JE, Andes DR, Mitchell AP. 2006. Function of *Candida albicans* adhesin Hwp1 in biofilm formation. *Eukaryot Cell* 5:1604–1610. <http://dx.doi.org/10.1128/EC.00194-06>.
34. Sudbery PE. 2011. Growth of *Candida albicans* hyphae. *Nat Rev Microbiol* 9:737–748. <http://dx.doi.org/10.1038/nrmicro2636>.
35. Mao X, Li Y, Wang H, Cao F, Chen J. 2008. Antagonistic interplay of *Swi1* and *Tup1* on filamentous growth of *Candida albicans*. *FEMS Microbiol Lett* 285:233–241. <http://dx.doi.org/10.1111/j.1574-6968.2008.01236.x>.
36. Lu Y, Su C, Mao X, Raniga PP, Liu H, Chen J. 2008. Efg1-mediated recruitment of NuA4 to promoters is required for hypha-specific *Swi1*/Snf binding and activation in *Candida albicans*. *Mol Biol Cell* 19:4260–4272. <http://dx.doi.org/10.1091/mbc.E08-02-0173>.
37. Lu Y, Su C, Wang A, Liu H. 2011. Hyphal development in *Candida albicans* requires two temporally linked changes in promoter chromatin for initiation and maintenance. *PLoS Biol* 9:e1001105. <http://dx.doi.org/10.1371/journal.pbio.1001105>.
38. Imanishi Y, Yokoyama K, Nishimura K. 2004. Inductions of germ tube and hyphal formations are controlled by mRNA synthesis inhibitor in *Candida albicans*. *Nihon Ishinkin Gakkai Zasshi* 45:113–119.
39. Kumar S, Chattopadhyay SK, Darokar MP, Garg A, Khanuja SP. 2007. Cytotoxic activities of xanthochymol and isoxanthochymol substantiated by LC-MS/MS. *Planta Med* 73:1452–1456. <http://dx.doi.org/10.1055/s-2007-990255>.
40. Al-Dhaheri RS, Douglas LJ. 2010. Apoptosis in *Candida* biofilms exposed to amphotericin B. *J Med Microbiol* 59:149–157. <http://dx.doi.org/10.1099/jmm.0.015784-0>.
41. Madeo F, Carmona-Gutierrez D, Ring J, Buttner S, Eisenberg T, Kroemer G. 2009. Caspase-dependent and caspase-independent cell death pathways in yeast. *Biochem Biophys Res Commun* 382:227–231. <http://dx.doi.org/10.1016/j.bbrc.2009.02.117>.
42. Cao Y, Huang S, Dai B, Zhu Z, Lu H, Dong L, Cao Y, Wang Y, Gao P, Chai Y, Jiang Y. 2009. *Candida albicans* cells lacking CaMCA1-encoded metacaspase show resistance to oxidative stress-induced death and change in energy metabolism. *Fungal Genet Biol* 46:183–189. <http://dx.doi.org/10.1016/j.fgb.2008.11.001>.
43. Sharma M, Manoharlar R, Puri N, Prasad R. 2010. Antifungal curcumin induces reactive oxygen species and triggers an early apoptosis but prevents hyphae development by targeting the global repressor *TUP1* in *Candida albicans*. *Biosci Rep* 30:391–404. <http://dx.doi.org/10.1042/BSR20090151>.
44. Cuesta-Rubio O, Frontana-Urbe BA, Ramirez-Apan T, Cardenas J. 2002. Polyisoprenylated benzophenones in Cuban propolis: biological activity of nemorosone. *Z Naturforsch C* 57:372–378.
45. Sogo AG, Heilmann CJ, Dekker HL, Bekker M, Brul S, de Koster CG, de Koning LJ, Klis FM. 2011. Effects of fluconazole on the secretome, the wall proteome, and wall integrity of the clinical fungus *Candida albicans*. *Eukaryot Cell* 10:1071–1081. <http://dx.doi.org/10.1128/EC.05011-11>.
46. Bujdaková H, Kulkova N, Cernakova L. 2012. Susceptibility to caspofungin and fluconazole and *Als1/Als3* gene expression in biofilm and dispersal cells of *Candida albicans*. *Arh Hig Rada Toksikol* 63:497–503. <http://dx.doi.org/10.2478/10004-1254-63-2012-2236>.
47. Walraven CJ, Bernardo SM, Wiederhold NP, Lee SA. 2014. Paradoxical antifungal activity and structural observations in biofilms formed by echinocandin-resistant *Candida albicans* clinical isolates. *Med Mycol* 52:131–139. <http://dx.doi.org/10.1093/mmy/myt007>.
48. Yang W, Yan L, Wu C, Zhao X, Tang J. 2014. Fungal invasion of epithelial cells. *Microbiol Res* 169:803–810. <http://dx.doi.org/10.1016/j.micres.2014.02.013>.
49. Wilson D, Mayer FL, Miramon P, Citiulo F, Slesiona S, Jacobsen ID, Hube B. 2014. Distinct roles of *Candida albicans*-specific genes in host-pathogen interactions. *Eukaryot Cell* 13:977–989. <http://dx.doi.org/10.1128/EC.00051-14>.
50. Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. 2012. *Candida albicans* morphogenesis and host defense: discriminating invasion from colonization. *Nat Rev Microbiol* 10:112–122. <http://dx.doi.org/10.1038/nrmicro2711>.
51. Berman J, Sudbery PE. 2002. *Candida albicans*: a molecular revolution built on lessons from budding yeast. *Nat Rev Genet* 3:918–930. <http://dx.doi.org/10.1038/nrg948>.