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ABSTRACT

Triclosan is a broad-spectrum antimicrobial compound commonly used in oral hygiene products. Investigation of its activity against *Candida albicans* showed that triclosan was fungicidal at concentrations of 16 mg/L. However, at subinhibitory concentrations (0.5–2 mg/L), triclosan antagonized the activity of fluconazole. Although triclosan induced *CDR1* expression in *C. albicans*, antagonism was still observed in *cdr1Δ* and *cdr2Δ* strains. Triclosan did not affect fluconazole uptake or alter total membrane sterol content, but did induce the expression of *FAS1* and *FAS2*, indicating that its mode of action may involve inhibition of fatty acid synthesis, as it does in prokaryotes. However, *FAS2* mutants did not exhibit increased susceptibility to triclosan, and overexpression of both *FAS1* and *FAS2* alleles did not alter triclosan susceptibility. Unexpectedly, the antagonistic effect was specific for *C. albicans* under hypha-inducing conditions and was absent in the non-filamentous *efg1Δ* strain. This antagonism may be due to the membranotropic activity of triclosan and the unique composition of hyphal membranes.

KEY WORDS: triclosan, fluconazole, antagonism, *Candida albicans*, *EFG1*, hyphae.

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Triclosan Antagonizes Fluconazole Activity against *Candida albicans*

INTRODUCTION

Triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol) is a small hydrophobic bisphenolic compound that exhibits a broad spectrum of antimicrobial activity (McDonnell and Russell, 1999). It is widely used in a variety of oral healthcare products, where it has been shown to possess potent anti-plaque activity (Marsh, 1991; Bhargava and Leonard, 1996). Triclosan is also commonly incorporated into soaps and plastics as an antimicrobial in both domestic and healthcare settings. In studies with *Escherichia coli*, *FabI* encoding a component of the fatty acid synthase machinery has been identified as the primary target of triclosan inhibition (McMurry *et al.*, 1998; Heath *et al.*, 1999). *FabI* encodes an NADH-dependent enoyl reductase that catalyzes the final reaction of the fatty acid elongation cycle.

Because of the pervasiveness of triclosan in the everyday environment, concerns have been raised about the safety of this compound (Levy, 2001). In particular, the role of triclosan in selecting for bacteria resistant to multiple drugs and antibiotics has become a concern (Chuanchuen *et al.*, 2001). However, the use of triclosan hand-washes has not been directly linked to changes in bacterial susceptibility to antibiotics (Aiello *et al.*, 2004). Although triclosan exhibits antifungal activity, few studies have examined the effects of this agent on *Candida albicans*, the major fungal pathogen of humans (Giuliana *et al.*, 1997; Yu *et al.*, 2011). *C. albicans* is a cause of oral and vaginal mucosal infections, commonly referred to as thrush. In critically ill patients, *C. albicans* can also cause life-threatening systemic infection. Since *C. albicans* is a common resident of the oral cavity, daily use of oral healthcare products containing triclosan would expose this organism to significant quantities of this agent. However, the interaction between triclosan and common azole antifungal drugs has not been fully investigated. In a recent study, it has been shown that triclosan can exhibit synergy with fluconazole against fluconazole-resistant *C. albicans* strains (Yu *et al.*, 2011). In this study, we investigated the activity of triclosan against azole-susceptible *C. albicans* and other common *Candida* species and identified an antagonistic interaction between triclosan and the azole antifungal drugs.

MATERIALS & METHODS

Strains and Growth Conditions

For routine strain maintenance, *Candida* strains (Appendix Table 1) were cultured in yeast extract peptone dextrose (YEPD) broth or agar at 37°C.

Figure 1. Susceptibility of *C. albicans* SC5314 in RPMI-1640 to fluconazole. Drug susceptibilities were tested by the EUCAST method. Fluconazole susceptibility of SC5314 was tested in RPMI-1640 medium in the absence and presence of triclosan (1, 2, and 4 mg/L). Dotted line indicates the IC_{50} and IC_{80} cut-offs as indicated. All plates were incubated at 37°C for 24 hrs, and growth was measured as absorbance at 540 nm. Results shown are the average of data generated in four separate experiments.

Fluconazole susceptibility was determined by broth microdilution (BMD) according to EUCAST Edef 7.1 (Rodríguez-Tudela *et al.*, 2008). The medium used for BMD was RPMI-1640 containing L-glutamine, buffered with MOPS and supplemented with 2% (w/v) glucose. To promote growth in the yeast phase, some BMD experiments were performed with yeast nitrogen base (YNB) medium without amino acids, supplemented with 2% (w/v) glucose. Where noted, media were supplemented with triclosan (TRC; Irgasan, Fluka, Buchs, Switzerland). IC_{50} s and IC_{80} s for fluconazole were defined as the concentration of drug that was required to inhibit growth by 50% or 80% relative to drug-free controls, respectively. The fractional inhibitory concentration index (ΣFIC) was calculated to determine whether drug interactions were antagonistic or synergistic according to the formula: $\Sigma FIC = (MIC \text{ fluconazole in triclosan} / MIC \text{ fluconazole alone}) + (MIC \text{ triclosan in fluconazole} / MIC \text{ triclosan alone})$ (Te Dorsthorst *et al.*, 2002; Rodríguez-Tudela *et al.*, 2008).

Fluconazole Uptake by Fungal Cells

Fluconazole uptake was determined in RPMI medium in the absence and presence of triclosan (1 mg/L) with [3H]-fluconazole [final concentration 50 nM (0.015 mg/L)] as previously described (Mansfield *et al.*, 2010). Fluconazole accumulation was also measured during batch growth with triclosan (1 mg/L) and [3H]-fluconazole, with measurements taken at 1, 3, and 24 hrs.

Analysis of Total Membrane Sterol Content in *C. albicans*

Total membrane sterols were isolated from cells grown in RPMI medium in the presence and absence of 1 mg/L triclosan, as

described previously (Martel *et al.*, 2010a). Derivatized sterols were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) and identified with reference to retention times and fragmentation spectra for known standards. Sterol chromatograms were analyzed with Agilent software (MSD Enhanced ChemStation, Agilent Technologies Inc., Palo Alto, CA, USA) for the derivation of integrated peak areas (Martel *et al.*, 2010b).

RNA Isolation and Quantitation

To facilitate isolation of large amounts of RNA, we grew cells under conditions identical to those used in the BMD assays, but in 50-mL volumes in 75 cm² polystyrene tissue culture flasks, and harvested them after 24 hrs. RNA was isolated and cDNA synthesized as described previously (O'Connor *et al.*, 2010). qRT-PCR was carried out in an ABI FAST7500 with SYBR Green (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Primers used in qRT-PCR are listed in Appendix Table 2, prefixed 'RT'. Expression of *FAS1*, *FAS2*, *CDR1*, and *CDR2* was measured. *ACT1* was included as an internal control, and all measurements were normalized against *ACT1* in each sample before comparison with other conditions. $2^{-\Delta\Delta CT}$ values were calculated according to Schmittgen and Livak (2008) and are represented graphically.

RESULTS

Triclosan Antagonizes Azole Activity against *C. albicans*

Triclosan was fungicidal against *C. albicans* at a concentration of 16 mg/L. Measurement of fluconazole MICs by the EUCAST broth microdilution assay showed that the addition of subinhibitory concentrations of triclosan (0.5–2.0 mg/L) to RPMI-1640 medium interfered with fluconazole antifungal activity against *C. albicans* (Fig. 1). The fluconazole IC_{50} in the absence of triclosan was 0.125 mg/L, which increased to 8 mg/L fluconazole in the presence of 1 mg/L triclosan. Calculation of the ΣFIC yielded a value of 64.25, which indicated an antagonistic interaction. This phenotype was confirmed with 8 additional *C. albicans* isolates (Appendix Table 1). In addition, the activity of other azoles (ketoconazole, itraconazole, and miconazole) against *C. albicans* was antagonized similarly by 1 mg/L triclosan, but the activity of amphotericin B was not affected (Appendix Fig. 1).

Expression of *CDR1* and *CDR2* in Response to Triclosan

Addition of 1 mg/L triclosan to the growth medium caused a significant increase in expression of *CDR1* in CAF2-1 (Fig. 2A). Triclosan exposure resulted in a small but non-significant decrease in *CDR2* mRNA levels in CAF2-1. To investigate whether changes in drug pump expression were directly involved in antagonism, we measured the IC_{80} of triclosan and the level of triclosan-mediated fluconazole antagonism in $\Delta cdr1$ and $\Delta cdr2$ mutants (Appendix Fig. 2). These mutations did not affect triclosan IC_{80} (Appendix Fig. 2A). Deletion of *CDR1* alone or in combination with *CDR2* caused increased susceptibility to fluconazole (IC_{80} was reduced to 0.25 mg/L compared

with 0.5 mg/L in the parental strain; Appendix Fig. 2B). However, mutation of the drug efflux pumps *CDR1* and *CDR2* did not eliminate the antagonism (Appendix Fig. 2B).

Fluconazole Accumulation Is Not Influenced by Triclosan

We assessed whether triclosan antagonized fluconazole activity by altering fluconazole uptake (Mansfield *et al.*, 2010). Addition of triclosan did not significantly affect fluconazole accumulation in CAF2-1 (Fig. 2B). Fluconazole accumulation was also measured in cells pre-grown in triclosan (1 mg/L) prior to cell starvation, or during batch growth with triclosan (1 mg/L) and [3 H]-fluconazole. No significant effect was observed in any condition (data not shown).

Membrane Sterol Content Is Not Affected by Triclosan

The membrane sterol contents of *C. albicans* cells exposed to triclosan (1 mg/L) in RPMI-1640 medium were investigated. No significant difference in sterol profile was identified in triclosan-treated and untreated cells (Appendix Table 3).

Alteration of FAS2 Levels Does Not Influence Triclosan Sensitivity

Triclosan exposure resulted in a drop in the expression levels of the fatty acid synthase encoding genes *FAS1* and *FAS2* by approximately 50% and 30%, respectively (Fig. 2A). To investigate whether Fas2p, the functional orthologue of the bacterial target FabI, is a possible target of triclosan inhibition, we investigated whether a heterozygous *FAS2/fas2Δ* mutant (CFD1) had altered triclosan susceptibility. Despite having only one copy of *FAS2*, CFD1 was found to have no alteration in triclosan MIC (Appendix Fig. 3A) or in triclosan-induced fluconazole antagonism (data not shown). We also overexpressed *FAS1* and *FAS2* in SC5314 using the pNIM1 doxycycline-inducible expression element (see Appendix for methods) (Park and Morschhauser, 2005). Overexpression of *FAS1* and *FAS2* from pNIM1 was confirmed by qRT-PCR and was reproducibly at least 2.0-fold greater at doxycycline concentrations ≥ 10 mg/L. Induction of *FAS1* or *FAS2* from the pNIM1 element with doxycycline (10–40 mg/L) did not reduce the triclosan susceptibility of SC5314 (Appendix Fig. 3B) or the antagonism of fluconazole (Appendix Fig. 4).

Antagonism of Fluconazole Activity Is Restricted to *C. albicans* Hyphae

Triclosan was fungicidal against *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. dubliniensis* at concentrations between 4 and 16 mg/L (Fig. 3A). Antagonism was not observed in *C. glabrata* (data not shown). *C. tropicalis* and *C. parapsilosis* isolates exhibited a 2- to 4-fold increase in fluconazole IC_{50} in the presence of 1 mg/L triclosan (Figs. 3B, 3C). However, the antagonistic effect at high fluconazole concentrations (> 8 mg/L) seen in *C. albicans* was not observed in either species. Unexpectedly, the closely related species *C. dubliniensis* (5 isolates, Appendix Table 1) exhibited a complete absence of fluconazole antagonism (Fig. 3D). Since *C. dubliniensis* grows exclusively in the yeast phase in RPMI medium, and *C. albicans* forms true

Figure 2. Analysis of fluconazole efflux and uptake. (A) Relative expression of *FAS1*, *FAS2*, *CDR1*, and *CDR2* in CAF2-1 and HLC52 ($\Delta efg1\Delta$) in the presence and absence of 1 mg/L triclosan. The upper and lower dashed lines indicate 2-fold increased and 2-fold decreased expression relative to CAF2-1, respectively. Cells were grown for 24 hrs on YEPD plates at 37°C and inoculated into RPMI-1640 at 2×10^5 cfu/mL, grown for 24 hrs at 37°C, and harvested. (B) [3 H]-Fluconazole accumulation by *C. albicans* strains, expressed as counts per min (CPM)/ 10^8 cells. Cells were grown in RPMI-1640 medium and washed, and the accumulation of [3 H]-fluconazole was measured following 24-hour incubation. Live and heat-killed SC5314 were included as positive and negative controls, respectively.

hyphae, we investigated whether morphology affected antagonism (Moran *et al.*, 2007; O'Connor *et al.*, 2010). Antagonism assays were repeated with a growth medium that promotes growth of *C. albicans* in the yeast phase (YNB, pH 5.6). *C. albicans* exhibited an identical triclosan IC_{50} in RPMI and YNB (16 mg/L) but exhibited a 4-fold higher fluconazole IC_{50} (0.5 mg/L) in YNB medium (Fig. 4A). However, antagonism of fluconazole activity by triclosan was not observed in YNB medium. To further explore the role of morphology in antagonism, we also examined antagonism in strain HLC52, which has a homozygous deletion in *EFG1* ($\Delta efg1\Delta$), a key regulator of hypha formation. Deletion of *EFG1* resulted in growth in the yeast form and greatly reduced antagonism in the presence 1 mg/L triclosan and fluconazole compared with the control

Figure 3. Sensitivity to triclosan (A) and fluconazole (B-D) of non-*albicans* *Candida* species. Drug susceptibilities were tested by the EUCAST method. Strains [*C. tropicalis* 3111 (B), *C. parapsilosis* HEM20 (C), and *C. dubliniensis* Wü284 (D)] were grown on YEPD plates at 37°C for 24 hrs before inoculation at 2×10^5 cfu/mL. Triclosan was added at 1 mg/L where indicated in panels B-D. Plates were incubated at 37°C for 24 hrs, and growth was measured as absorbance at 540 nm. Results are the average of at least 3 independent experiments. Dotted lines on Y-axes indicate IC_{50} values.

CAF2-1 (Figs. 4B, 4C). Complementation of $\Delta efg1$ with a single copy of *EFG1* (strain HLCEFG) restored antagonism (Fig. 4D). Analysis of gene expression showed that the $\Delta efg1$ mutant exhibited a significant increase in expression of both *FAS1* and *FAS2* compared with CAF2-1 (Fig. 2). In addition, *CDR2* expression was constitutively high compared with CAF2-1, even in the absence of triclosan. Analysis of fluconazole uptake in $\Delta efg1$ cells indicated that they accumulated less fluconazole than CAF2-1; however, the levels were not affected by the addition of triclosan (Fig. 2).

DISCUSSION

Triclosan is commonly used as an anti-plaque agent and displays a high level of oral retention in plaque and on tooth surfaces for several days following administration (Creeth *et al.*, 1993). We observed that, against *C. albicans*, subinhibitory triclosan concentrations (0.5-2 mg/L) could antagonize the activity of fluconazole. Although triclosan accumulates to high concentrations in plaque, its aqueous solubility is < 10 mg/L (Loftsson *et al.*, 1999). Triclosan is therefore unlikely to reach IC_{50} concentrations in saliva or other bodily fluids for extended

periods, and residual concentrations in saliva and plasma are within the range of antagonistic triclosan concentrations identified here (Creeth *et al.*, 1993; Lin, 2000; Calafat *et al.*, 2008).

A recent study by Yu *et al.* reported synergy between fluconazole and triclosan against fluconazole-resistant *C. albicans* isolates, but did not report the effects of this compound on azole-susceptible yeasts (Yu *et al.*, 2011). These authors did not detect the antagonism described here because of the intrinsic fluconazole resistance of the isolates studied ($IC_{50}s > 16 \mu\text{g/mL}$). As such, the effects of triclosan on fluconazole MIC described here would not have been apparent in these isolates.

To elucidate its mechanism, we carried out a detailed study of triclosan-mediated fluconazole antagonism. The possibility that this phenomenon could be due to a physical interaction between the two drugs could be excluded, since the antagonism was not observed in non-*albicans* *Candida* species. Our data exclude changes in membrane sterol content, altered drug efflux, or altered uptake as mechanisms of triclosan-induced azole antagonism. Our data also exclude a role for the fungal orthologue of FabI, encoded by *FAS2* in *C. albicans*, in the mode of action of triclosan. Strains exhibiting increased or decreased expression of *FAS2* did not exhibit altered susceptibility to

Figure 4. Fluconazole antagonism in *C. albicans* requires hypha formation. Drug susceptibilities were tested by the EUCAST method with YNB (A) or RPMI (B–D). Strains SC5314, CAF2-1, and HLC52 ($\Delta efg1$) were grown on YEPD plates at 37°C for 24 hrs before inoculation at 2×10^5 cfu/mL. Triclosan was added at 1 mg/L (+ TRC) unless otherwise indicated. Plates were incubated at 37°C for 48 hrs, and growth was measured as absorbance at 540 nm. Results are the average of at least three independent experiments. Dotted lines on Y-axes indicate IC_{50} or IC_{80} values as indicated.

triclosan or antagonism. From these studies, we concluded that *FAS2* was unlikely to be the major target of triclosan in *C. albicans*. Since these data were generated, the crystal structure of the Fas2 enzyme from *S. cerevisiae* has been determined at high resolution, and it was concluded that triclosan is unlikely to bind to the enoyl reductase active site, supporting the genetic evidence presented here (Jenni *et al.*, 2007).

One unexpected observation from these studies was that antagonism was specific for the hyphal form of *C. albicans*. The hyphal form of *C. albicans* is highly adherent and invasive, and triclosan antagonism may therefore allow invasive fungal infections to persist. Antagonism was not observed in YNB medium, which at 30°C restricts *C. albicans* to the yeast morphology, or in the *efg1 Δ mutant HLC52, which is unable to form hyphae in RPMI-1640 medium. Although the *efg1 Δ mutant exhibited deregulated expression of *FAS1* and *FAS2*, analysis of our data indicated that fatty acid synthases are unlikely to be the targets of triclosan in *C. albicans*.**

Since our data exclude the involvement of many specific targets in the mode of action of triclosan (fatty acid synthases, sterol metabolism, CDR mechanisms), we hypothesize that triclosan may act as a non-specific membranotropic agent against *C. albicans*, mediating non-specific damage to the plasma membrane that accounts for its fungicidal and antagonistic activities. Recent research has re-appraised the role of membrane intercalation by triclosan as part of its biocidal action (Villalain *et al.*, 2001; Lygre *et al.*, 2003; Guillen *et al.*, 2004). At low concentrations, triclosan has been shown to alter bacterial membrane fluidity and function without actually causing cell lysis, whereas at higher concentrations cell lysis may occur (Regos *et al.*, 1979; Villalain *et al.*, 2001). In *C. albicans*, subinhibitory concentrations of triclosan (≤ 8 mg/L) could also induce changes in membrane fluidity, and this could be the cause of fluconazole antagonism, perhaps by counteracting the disruptive effects of toxic sterols. Indeed, fluconazole resistance has previously been associated with increased membrane fluidity (Kohli *et al.*,

2002). The different activities of triclosan at subinhibitory concentrations in yeast and hyphal cells may also be related to altered membrane content and fluidity (Prasad *et al.*, 2010). The *efg1Δ* mutant has a significantly different lipid composition compared with the wild-type, and exhibits decreased membrane fluidity and increased fluconazole accumulation. We can only hypothesize at this stage that changes in membrane content and fluidity in hyphal cells result in a different interaction with triclosan compared with yeasts.

This study raises concerns about the concurrent use of triclosan-containing products and azole antifungals. The widespread use of triclosan in everyday hygiene and oral healthcare products makes it highly likely that infecting *C. albicans* strains are regularly exposed to this agent. How this affects antifungal therapy in these patients has yet to be explored, and further investigations will be required to determine whether this interaction is clinically significant.

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