Triclosan antagonises fluconazole activity against *Candida albicans*

Judy Higgins[1], Emmanuelle Pinjon[1], Hanna N. Oltean[2], Theodore C. White[2], Steve L. Kelly[3], Claire M. Martel[3], Derek J. Sullivan[1], David C. Coleman[1], Gary P. Moran[1]*

[1]Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental University Hospital, University of Dublin, Trinity College Dublin, Dublin 2, Ireland; [2]Seattle Biomedical Research Institute, Seattle, Washington, USA; [3]Institute of Life Science and School of Medicine, Swansea University, Swansea, SA2 8PP, Wales, United Kingdom.

*Corresponding author: Dublin Dental University Hospital, University of Dublin, Trinity College Dublin, Lincoln Place, Dublin 2, Ireland. Tel: +353 16127245, e-mail: gpmoran@dental.tcd.ie.

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 *cdr1*Δ and *cdr2*Δ 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 nonfilamentous *efg1*Δ strain. This antagonism may be due to the membranotropic activity of triclosan and the unique compostion of hyphal membranes.

Keywords: triclosan, fluconazole, antagonism, *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.

Due to 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. As *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 and 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. Fluconazole susceptibility was determined by broth microdilution (BMD) according to EUCAST Edef 7.1 (Rodriguez-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). 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 (∑FIC) was calculated to determine whether drug interactions were antagonistic or synergistic according to the formula: ∑FIC = (MIC fluconazole in triclosan/ MIC fluconazole alone) + (MIC triclosan in fluconazole/ MIC triclosan alone)(Te Dorsthorst et al., 2002; Rodriguez-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) using [^{3}H]-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 [^{3}H]-fluconazole, with measurements taken at 1 h, 3 h and 24 h.

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

RNA isolation and quantitation

To facilitate isolation of large amounts of RNA, cells were grown under conditions identical to those used in the BMD assays, but in 50 ml volumes in 75 cm^2 polystyrene tissue culture flasks, and harvested after 24 h. RNA was isolated and cDNA synthesised as described (O'Connor et al., 2010). qRT-PCR was carried out in an ABI FAST7500 using SYBR Green (Applied Biosystems, Warrington, United Kingdom) according to manufacturer's instructions. Primers used in qRT-PCR are listed in the 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 normalised against *ACT1* in each sample before comparison with other conditions. $2^{-\Delta\Delta C}{}_T$ values were calculated according to Schmittgen and Livak (Schmittgen and Livak, 2008) and represented graphically.

Results

Triclosan antagonises azole activity against Candida albicans

Triclosan was fungicidal against *C. albicans* at a concentration of 16 mg/L. Measurement of fluconazole MICs by the EUCAST broth micodilution 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 ∑FIC 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 antagonised in a similar way 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 Δ*cdr1* and Δ*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 to 0.5 mg/L in the parental strain; Appendix Fig. 3A). However, mutation of the drug efflux pumps *CDR1* and *CDR2* did not eliminate the antagonism (Appendix Fig. 3A).

Fluconazole accumulation is not influenced by triclosan

We assessed whether triclosan antagonised fluconazole activity by altering fluconazole uptake (Mansfield et al., 2010). Addition of triclosan did not significantly effect fluconazole accumulation in CAF2-1 (Fig. 2B). Fluconazole accumulation was also measured in cells pregrown 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 content 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). In order 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/fas2*Δ 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. 3). 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 (Fig. 3B and C). 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. dublinensis* (5 isolates, Appendix Table 1) exhibited a complete absence of fluconazole antagonism (Fig. 3D). As *C. dubliniensis* grows exclusively in the yeast phase in RPMI medium and *C. albicans* forms true hyphae, we investigated whether morphology affected antagonism (Moran et al., 2007; O'Connor et al., 2010). Antagonism assays were repeated using 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* (Δ*efg1*), 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 to the control CAF2-1 (Fig. 4B and C). Complementation of Δ*efg1* with a single copy of *EFG1* (strain HLCEFG) restored antagonism (Fig. 4D). Analysis of gene expression showed that the Δ*efg1* mutant exhibited a significant increase in expression of both *FAS1* and *FAS2* compared to CAF2-1

199 (Fig. 2). In addition, *CDR2* expression was constitutively high compared to CAF2-1 even in
200 the absence of triclosan. Analysis of fluconazole uptake in Δ*efg1* cells indicated that they
201 accumulated less fluconazole than CAF2-1, however the levels were not affected by the
202 addition of triclosan (Fig. 2).
203

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). Yu et al. did not detect the antagonism described here due to the intrinsic fluconazole resistance of the isolates studied (IC_{50}s >16 μg/ml). As such, the effects of triclosan on fluconazole MIC described here would not have been apparent in these isolates.

We carried out a detailed study of triclosan-mediated fluconazole antagonism in order to elucidate its mechanism. The possibility that this phenomenon could be due to a physical interaction between the two drugs could be excluded, as 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 excludes 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 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

228 determined at high resolution and it was concluded that triclosan is unlikely to bind to the
229 enoyl reductase active site, supporting the genetic evidence presented here (Jenni et al.,
230 2007).

231 One unexpected observation from these studies was that antagonism was specific for
232 the hyphal form of *C. albicans*. The hyphal form of *C. albicans* is highly adherent and
233 invasive and triclosan antagonism may therefore allow invasive fungal infections to persist.
234 Antagonism was not observed in YNB medium, which at 30˚C restricts *C. albicans* to the
235 yeast morphology, or in the *efg1*Δ mutant HLC52, which is unable to form hyphae in RPMI-
236 1640 medium. Although the *efg1*Δ mutant exhibited deregulated expression of *FAS1* and
237 *FAS2*, our data indicate that fatty acid synthases are unlikely to be the targets of triclosan in
238 *C. albicans*.

239 As our data excludes the involvement of many specific targets in the mode of action of
240 triclosan (fatty acid synthases, sterol metabolism, CDR mechanisms), we hypothesize that
241 triclosan may act as a non-specific membranotropic agent against *C. albicans*, mediating non-
242 specific damage to the plasma membrane that accounts for its fungicidal and antagonistic
243 activities. Recent research has reappraised the role of membrane intercalation by triclosan as
244 part of its biocidal action (Villalain et al., 2001; Lygre et al., 2003; Guillen et al., 2004). At
245 low concentrations, triclosan has been shown to alter bacterial membrane fluidity and
246 function without actually causing cell lysis, whereas at higher concentrations cell lysis may
247 occur (Regos et al., 1979; Villalain et al., 2001). In *C. albicans*, subinhibitory concentrations
248 of triclosan (≤ 8 mg/L) could also induce changes in membrane fluidity and this could be the
249 cause of fluconazole antagonism, perhaps by counteracting the disruptive effects of toxic
250 sterols. Indeed, fluconazole resistance has previously been associated with increased
251 membrane fluidity (Kohli et al., 2002). The different activity of triclosan at subinhibitory
252 concentrations in yeast and hyphal cells may also be related to altered membrane content and

253 fluidity (Prasad et al., 2010). The *efg1*Δ mutant has a significantly different lipid composition
254 compared to wild-type, exhibits decreased membrane fluidity and increased fluconazole
255 accumulation. We can only hypothesize at this stage that changes in membrane content and
256 fluidity in hyphal cells results in a different interaction with triclosan compared to yeasts.
257 This study raises concerns about the concurrent use of triclosan containing products and
258 azole antifungals. The widespread use of triclosan in everyday hygiene and oral healthcare
259 products makes it highly likely that infecting *C. albicans* strains are regularly exposed to this
260 agent. How this impacts on antifungal therapy in these patients has yet to be explored and
261 further investigations will be required to determine whether this interaction is clinically
262 significant.
263

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Transparency declaration

The authors have no conflicts of interest to declare.

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Figure Legends

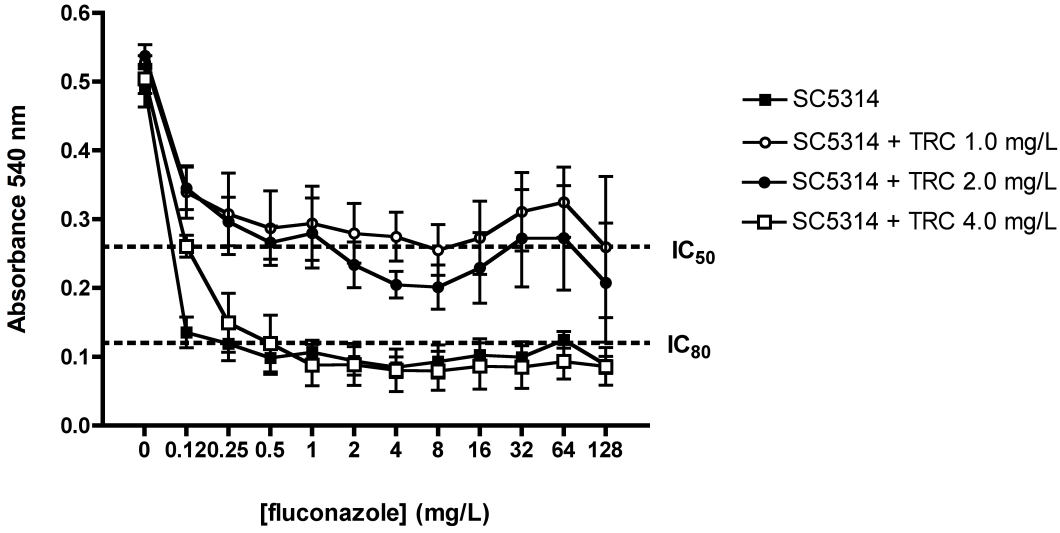
Figure 1. Susceptibility of *C. albicans* SC5314 in RPMI-1640 to fluconazole. Drug susceptibilities were tested using the EUCAST method. Fluconazole susceptibility of SC5314 was tested in RPMI-1640 medium in the absence and presence of triclosan (1, 2 and 4 mg/L). Dotted line indicates the IC_{50} and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

Figure 2. Analysis of fluconazole efflux and uptake (A) Relative expression of *FAS1, FAS2, CDR1* and *CDR2* in CAF2-1 and HLC52 (Δ*efg1*) 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 h on YEPD plates at 37˚C and inoculated into RPMI-1640 at 2 x 10^5 cfu/ml, grown for 24 h at 37˚C and harvested. (B) [^{3}H] Fluconazole accumulation by *C. albicans* strains, expressed as counts per minute (CPM)/10^8 cells**.** Cells were grown in RPMI-1640 medium, washed and the accumulation of [^{3}H]fluconazole was measured following 24 h incubation. Live and heat-killed SC5314 were included as positive and negative controls, respectively.

Figure 3. Sensitivity to triclosan (A) and fluconazole (B-D) of non-*albicans Candida* species. Drug susceptibilities were tested using 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 h before inoculation at 2 x 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 h and growth measured

391 as absorbance at 540 nm. Results are the average of at least three independent experiments.
392 Dotted lines on Y-axes indicate IC_{50} values.
393
394 **Figure 4. Fluconazole antagonism in *C. albicans* requires hypha formation.** Drug
395 susceptibilities were tested using the EUCAST method using YNB (A) or RPMI (B-D).
396 Strains SC5314, CAF2-1 and HLC52 (Δ*efg1*) were grown on YEPD plates at 37˚C for 24 h
397 before inoculation at 2 x 10^5 cfu/ml. Triclosan was added at 1 mg/L (+ TRC) unless
398 otherwise indicated. Plates were incubated at 37˚C for 48 h and growth measured as
399 absorbance at 540 nm. Results are the average of at least three independent experiments.
400 Dotted lines on Y-axes indicate IC_{50} or IC_{80} values as indicated.
401

Fig. 1


Absorbance 540 nm
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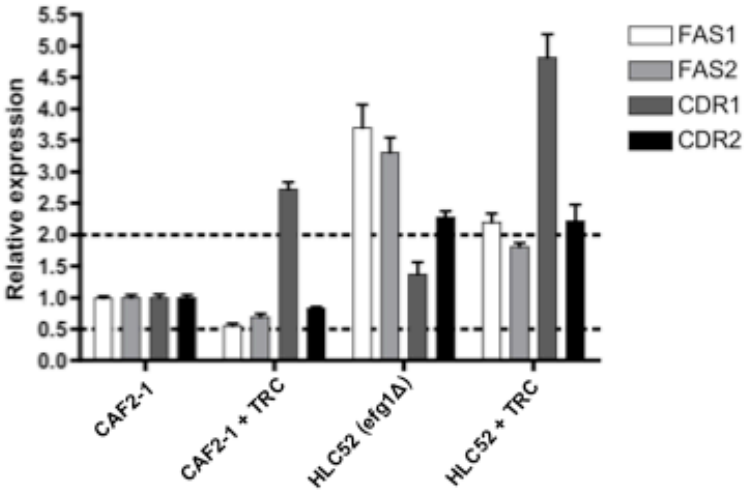
0.5
0.4
0.3
0.2
0.1
0.0
0 0.12 0.25 0.5 1 2 4 8 16 32 64 128
[fluconazole] (mg/L)
IC50
IC80
SC5314
SC5314 + TRC 1.0 mg/L
SC5314 + TRC 2.0 mg/L
SC5314 + TRC 4.0 mg/L

Fig. 2

A


Relative expression
5.5
5.0
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1.5
1.0
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0.0
FAS1
FAS2
CDR1
CDR2
CAF2-1
CAF2-1 + TRC
HLC52 (efg1Δ)
HLC52 + TRC

B

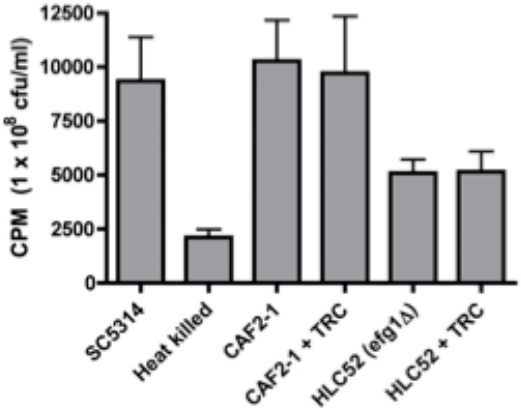

CPM (1 x 10^8 cfu/ml)
12500
10000
7500
5000
2500
0
SC5314
Heat killed
CAF2-1
CAF2-1 + TRC
HLC52 (efg1Δ)
HLC52 + TRC

Fig. 3

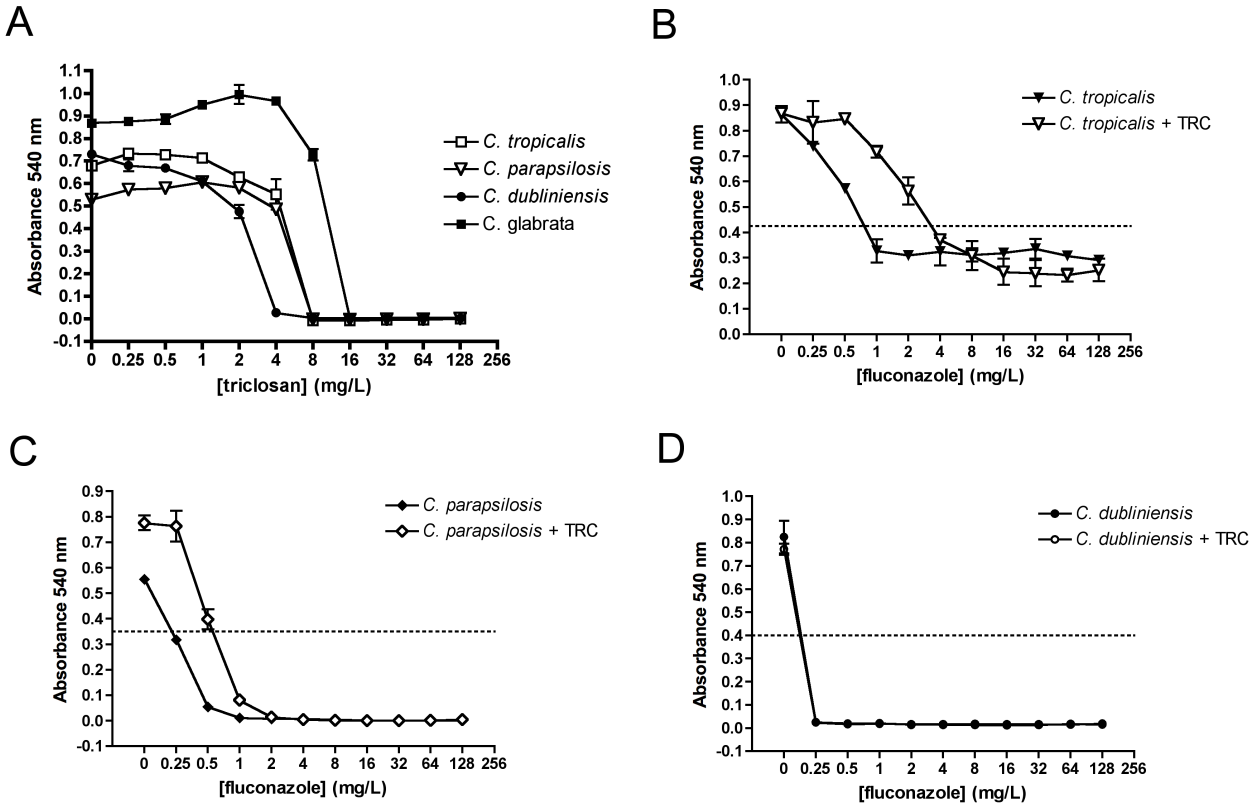
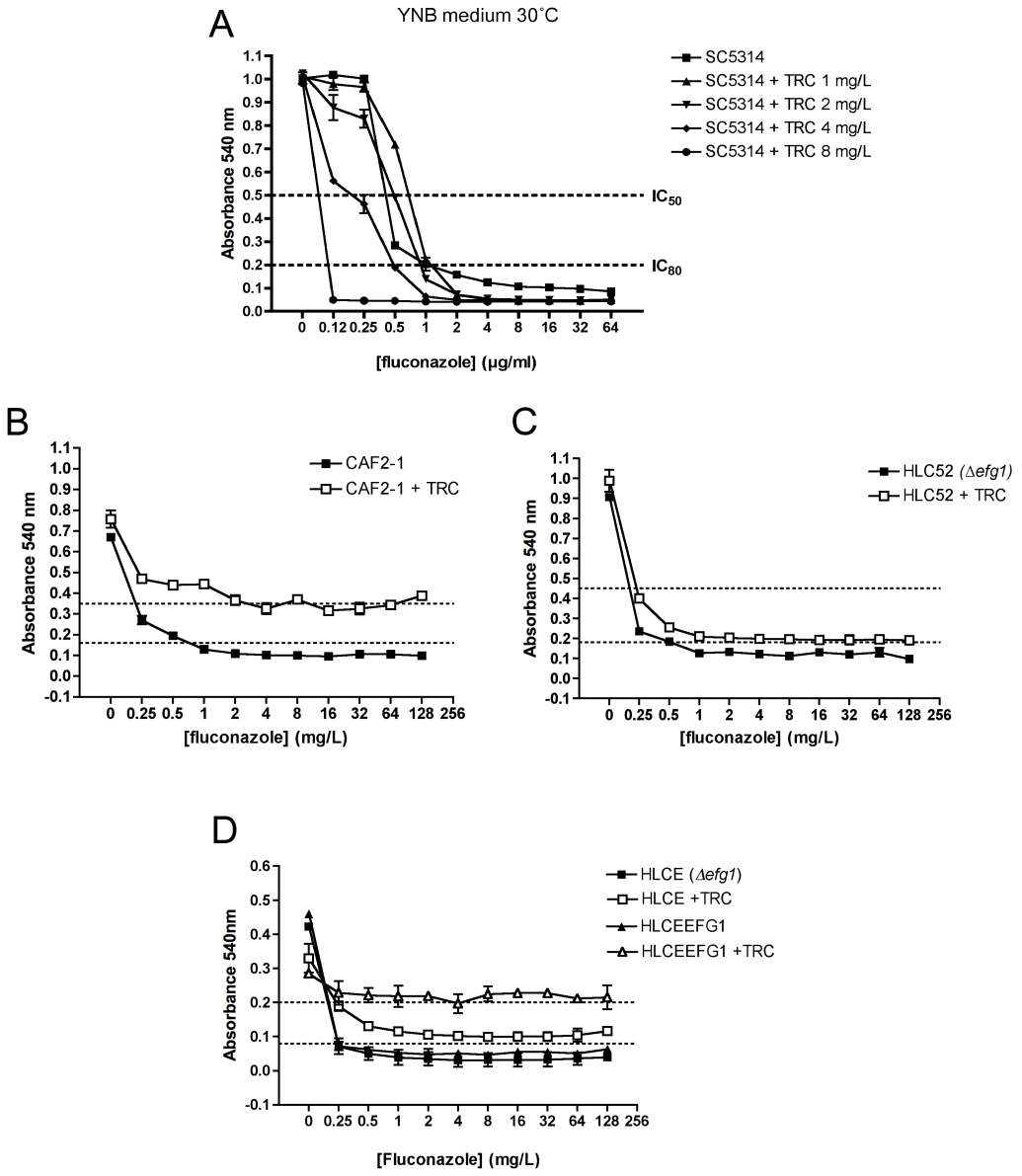

A
Absorbance 540 nm
[triclosan] (mg/L)
C. tropicalis
C. parapsilosis
C. dubliniensis
C. glabrata
B
Absorbance 540 nm
[fluconazole] (mg/L)
C. tropicalis
C. tropicalis + TRC
C
Absorbance 540 nm
[fluconazole] (mg/L)
C. parapsilosis
C. parapsilosis + TRC
D
Absorbance 540 nm
[fluconazole] (mg/L)
C. dubliniensis
C. dubliniensis + TRC

Fig. 4


A
YNB medium 30˚C
SC5314
SC5314 + TRC 1 mg/L
SC5314 + TRC 2 mg/L
SC5314 + TRC 4 mg/L
SC5314 + TRC 8 mg/L
IC50
IC80
Absorbance 540 nm
[fluconazole] (µg/ml)
B
CAF2-1
CAF2-1 + TRC
Absorbance 540 nm
[fluconazole] (mg/L)
C
HLC52 (Δefg1)
HLC52 + TRC
Absorbance 540 nm
[fluconazole] (mg/L)
D
HLCE (Δefg1)
HLCE +TRC
HLCEEFG1
HLCEEFG1 +TRC
Absorbance 540nm
[Fluconazole] (mg/L)