Tetracycline alters drug susceptibility in *Candida albicans* and other pathogenic fungi

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The tetracycline (TET) promoter has been used in several systems as an inducible regulator of gene expression. In control analyses, the standard *Candida albicans* laboratory strain SC5314 was found to have altered susceptibility to a variety of antifungal drugs in the presence of relatively high concentrations (50–200 μg ml⁻¹) of TET. Altered susceptibility was most notable with exposure to amphotericin B (AMB), with a 32-fold increase in susceptibility, and terbinafine (TRB), with a 32-fold decrease in susceptibility. The TET/AMB synergy was observed in several clinical isolates of *C. albicans* and in the distantly related species *Aspergillus fumigatus* and *Cryptococcus neoformans*. The TET/AMB synergy is not related to efflux pump activity, as determined by FACS analyses and by analysis of a strain containing efflux pump deletions. Gene expression analyses by luciferase and by quantitative real-time reverse transcriptase PCR failed to identify significant alterations in expression of any genes associated with resistance. *C. albicans* grown with TET for 48 h does show a reduction in total cellular ergosterol. Analysis of growth curves suggests that the TET effect is associated with lack of a diauxic shift, which is related to a loss of mitochondrial function. MitoTracker fluorescent dye was used to demonstrate related to a loss of mitochondrial function. These results demonstrate the need for careful analysis of TET effects when using a TET-inducible promoter, especially in studies that involve antifungal drugs. This study defines some limits to the use of the TET-inducible promoter, and identifies effects on cells that are the result of TET exposure alone, not the result of expression of a targeted gene.

INTRODUCTION

Tetracycline (TET), an antibiotic product of bacteria of the genus *Streptomyces*, has been in use since the 1950s (Chopra & Roberts, 2001). The mechanism of action for TET and its derivitive doxycycline (DOX) is the inhibition of translation through binding to the bacterial 30S ribosomal unit. This specificity for a bacterial component has led to an expectation that TET does not affect eukaryotic cells. However, TET is also known to affect protein synthesis in the mitochondria of eukaryotic cells (Chopra & Roberts, 2001), since the mitochondrial ribosome is related in structure and function to bacterial ribosomes. TET is known to have an effect on infections with several eukaryotic protozoan parasites including *Plasmodium falciparum*, *Entamoeba histolytica*, *Giardia lamblia*, *Leishmania major*, *Trichomonas vaginalis* and *Toxoplasma gondii*. Despite this, a TET-inducible system has been used in several protozoan systems including *Trichomonas vaginalis* (Ortiz & Johnson, 2003), *G. lamblia* (Sun et al., 2005), *Trypanosoma* species (Wirtz & Clayton, 1995) and *Leishmania* species (Yan et al., 2001). Recently TET has been used as an inducible promoter in fungal species such as *Candida albicans* (Park & Morschhauser, 2005), *Candida glabrata* (Nakayama et al., 1998), *Schizosaccharomyces pombe* (Faryar & Gatz, 1992) and *Aspergillus fumigatus* (Vogt et al., 2005). In most of these systems, genes with an inducible TET promoter are constitutively expressed in the absence of TET, while increasing levels of TET reduce expression of the target gene.

In the last 25 years, the incidence of fungal infections in the general population has increased dramatically due to an increase in the number of patients living in an immuno-compromised state (White, 1997; White et al., 1998).
Candida species with both intrinsic and acquired azole drug resistance are a leading cause of fungal infection within the immuno-compromised population (White et al., 1998). Several genes are commonly overexpressed in C. albicans azole-resistant strains. These genes are the efflux pump genes MDR1, CDR1 and CDR2, as well as the ergosterol biosynthetic pathway gene ERG11 (which is the target of azole drugs).

Treatment of fungal infections for the last 50 years has relied on fungicidal polyene drugs such as amphotericin B (AMB) that bind to the major fungal sterol, ergosterol. More recently, fungistatic drugs such as the azoles [fluconazole (FLC), itraconazole (ITC)] and the allylamines [terbinafine (TRB)] have become more broadly used to treat fungal infection due to the comparative ease of their use. Both the allylamines and azoles target genes in the ergosterol biosynthetic pathway. Until very recently, the gold standard treatment for fungal infections was AMB, and it remains a significant treatment option.

This laboratory is investigating the effect of altered expression of genes in the ergosterol biosynthetic pathway genes on antifungal susceptibility. To accomplish this, strains were acquired from other investigators in which genes in this pathway are linked to the TET promoter, allowing a reduction of gene expression in the presence of TET. However, exposure to TET in the control strains showed altered susceptibility to antifungal drugs at high TET concentrations.

In the 1970s and 1980s, synergy between TET and AMB was observed clinically and in animal models with wild-type strains of C. albicans, Aspergillus species and Cryptococcus species (Graybill & Mitchell, 1980; Kwan et al., 1972; Odds et al., 1986; Raab & Hogl, 1980; Rubin et al., 1983). However, no molecular mechanism was elucidated at the time for any of these species and it is important to revisit this phenomenon as the use of TET-regulatable promoters in these species has become increasingly common and as standardized methods of determining MIC have been defined. This study investigates susceptibilities and molecular mechanisms associated with exposure to TET and its synergy with AMB.

**METHODS**

**Strains.** The C. albicans strains used in this study were obtained as follows: SC5314 (W. Fonzi, Georgetown University), DSY1050 (D. Sanglard, University Hospital Lausanne), 90028 and 3153A (ATCC, Manassas, VA), and 2-76 and FH1 (our collection). Strains from other fungal species include H99 (Jennifer Lodge, St Louis University). Unless otherwise noted, strains were grown in RPMI 1640 at 30°C with shaking in the dark (to minimize TET degradation).

**Susceptibility testing.** Drug susceptibility was determined using the Clinical and Laboratory Standards Institute (CLSI) microbroth dilution protocol, which determines the MIC of drug needed to inhibit cell growth (NCCLS, 2002). Cell growth was measured using a Beckman DU 640B spectrophotometer at 600 nm (Beckman Coulter). MICs are reported using 20% relative growth (MIC<sub>90</sub>) at 48 h. Isolates were grown in a 96-well plate containing a gradient of drug, and in four concentrations of TET (300, 200, 100 and 50 µg ml<sup>-1</sup>). Etests were performed according to the manufacturer’s directions (AB Biodisk) in the presence and absence of TET in RPMI 1640 agar.

**Luciferase assays.** Luciferase assays were performed as indicated previously (Harry et al., 2005). Strains containing luciferase linked to full-length promoters of MDR1 (Harry et al., 2005), ERG11 (Song et al., 2004) or UPC2 (J. S. Hoot & T. C. White, unpublished observations) were grown in the presence or absence of either 20 or 200 µg TET ml<sup>-1</sup> for 48 h. Luciferase assays were performed during this time at 5 h, 24 h and 48 h.

**Ergosterol scans.** Ergosterol levels were measured using a protocol described previously (Arthlington Skaggs et al., 1999). Strains were grown for 48 h, and 300 OD<sub>600</sub> units of cells [concentration of cells (OD<sub>600</sub> ml<sup>-1</sup>) × volume (ml) = 300 OD<sub>600</sub> units] were used in heptane extraction of ergosterol at 24 h and 48 h. Ergosterol levels were determined on the spectrophotometer, scanning from 230 nm to 310 nm.

**FACS analysis for pump activity.** Cells were inoculated at OD<sub>600</sub> 0.2 and grown for 48 h in YEPD + TET (0, 50, 100, 200, 300 µg ml<sup>-1</sup>) in the dark at 30°C with shaking. After washing, the OD<sub>600</sub> of each culture was adjusted to 0.1. A 1 ml aliquot of each sample was removed to serve as a blank. The remaining cultures were spun down and resuspended in 50 nm rhodamine-123 (R123, Sigma) or 100 µm rhodamine 6G (R6G, Sigma) in water. Tubes were incubated in the dark at 30°C with shaking. Samples were removed at specified time points and analysed by flow cytometry using a Beckman Coulter Epics XL-MCL 4-colour cell analyser. The geometric mean of the fluorescence of each sample was calculated using FlowJo software.

**QRT-PCR.** Quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR) was performed using standard protocols. In brief, cultures were grown in the presence and absence of TET. RNA was prepared using the Qiagen RNeasy mini kit. Genomic DNA was removed by treatment with RQ1 DNase followed by heat inactivation. The RNAs were then reverse transcribed using the Quantitect Reverse Transcription kit (Qiagen). The CDNs were subsequently analysed in quantitative PCR using a SYBR green mastermix in an ABI 7500 (Applied Biosystems) following the manufacturer’s recommended protocols.

**MitoTracker analyses.** Cells were grown overnight in YAD (containing, per litre, 1.7 g yeast nitrogen base without ammonium sulfate, 5 g ammonium sulfate and 10 g glucose [dextrose]) at 30°C with shaking at 180 r.p.m. Cells were diluted to an OD<sub>600</sub> of 0.1 in 10 ml YAD, YAD plus 200 µg TET ml<sup>-1</sup> and YAD plus 600 µM sodium azide (NaN₃). TET- and NaN₃-treated cells were maintained in drug until fixation. After dilution, cells were incubated for 6 h at 30°C, with 180 r.p.m. shaking in the dark. Cells were collected by centrifugation and resuspended in fresh medium with the mitochondrial-specific dye MitoTracker Red CMXRos (MTR, Invitrogen) at a final concentration of 20 nM. Cells were incubated for 15 min at 30°C, 180 r.p.m. in the dark then washed once in fresh media. Stained cells were collected by centrifugation and resuspended in fresh media with 3.7% formaldehyde. Cells were incubated for 15 h at 30°C, 180 r.p.m. in the dark. Stained fixed cells were washed three times with PBS and immediately observed using a DeltaVision RT optical/digital-sectioning microscope equipped with DIC optics for visible imaging. Images were deconvolved and compiled as projections of digital sections using softWoRx Explorer 1.2 software. All images were processed identically.
Cells for FACS analysis were prepared as previously described for microscopy. Stained, fixed cells were immediately analysed using a Beckman Coulter Epics XL-MCL 4-colour cell analyser. Ten thousand cells per sample were analysed. The geometric mean of FL3 channel fluorescence for each sample was calculated using FlowJo software 7.2.2 (Tree Star).

RESULTS

Use of TET to regulate gene expression in eukaryotic cells is becoming increasingly common. In preliminary experiments using TET to regulate C. albicans genes involved in ergosterol biosynthesis, control experiments were performed using TET with the standard laboratory strain SC5314. These experiments demonstrated that TET had an effect on drug susceptibility testing, independent of the phenotype that might result from the downregulation of genes in the ergosterol biosynthesis pathway. Further investigation of this inhibition revealed a C. albicans susceptibility to TET with a MIC\(_{50}\) of 400 \(\mu\)g ml\(^{-1}\), which is relatively high but measurable.

**TET and DOX effect on AMB susceptibility**

The effect of TET was investigated on the relative growth of cells in the presence of AMB, using the standard laboratory strain SC5314 (Fig. 1a). Relative growth of cells with AMB was reduced with increasing concentrations of TET. This effect was titratable, from 50 to 300 \(\mu\)g ml\(^{-1}\), values that are below the 400 \(\mu\)g ml\(^{-1}\) MIC for TET.

While TET is commonly used in vitro in gene expression studies, DOX is commonly used clinically and in animal studies, where it induces the TET promoter. In vivo, DOX also resulted in increasing susceptibility to AMB (Fig. 1b).

**TET effect on MICs**

In C. albicans, the MIC is defined as the amount of drug that inhibits relative growth to 20\% (80\% inhibition), denoted as MIC\(_{50}\). The MIC\(_{50}\) values for the data from Fig. 1(a, b) are presented in Table 1 (rows 1 and 8). For both TET and DOX in combination with AMB, the MIC\(_{50}\) was reduced 32-fold for TET or DOX concentrations of 300 \(\mu\)g ml\(^{-1}\). Thus TET and DOX amplify the efficacy of AMB, increasing the susceptibility of cells to AMB.

The TET effect was evaluated for other drugs. FLC inhibits Erg11p, the product of ERG11 – one of the genes involved in ergosterol biosynthesis. Exposure to TET (to 300 \(\mu\)g ml\(^{-1}\)) increases the MIC\(_{50}\) of FLC for SC5314 fourfold (Table 1, row 2). Terbinafine (TRB) inhibits ERG1, a gene upstream of ERG11 in the ergosterol biosynthesis pathway. TET (200–300 \(\mu\)g ml\(^{-1}\)) increases the MIC\(_{50}\) 32-fold (Table 1, row 3), making the cells more resistant to TRB. Thus TET increases the resistance to two drugs (FLC, TRB) that target ergosterol biosynthesis pathway genes while increasing the susceptibility to a drug (AMB) that targets the end product of the pathway, ergosterol.

Drugs that are not standard antifungals were also tested. DTT is a denaturing agent that disrupts cell-wall stability in part because it is a reducing agent. High concentrations of TET increase the resistance to DTT (Table 1, row 4) 32-fold, suggesting a link between ergosterol and the effect of DTT on cell-wall stability or on the redox state of the cells (see below). However, SDS disrupts membranes and cell walls, and TET had no effect on SDS (Table 1, row 5). Calcofluor white (CFW) also interferes with cell-wall stability and function, and TET had no effect on susceptibility to CFW (Table 1, row 6). Finally, caspofungin (CSP) is a clinically useful antifungal that targets glucan synthase in the cell membrane and inhibits the synthesis of the 1,3-\(\beta\)-glucans in the cell wall. There was no TET effect on the CSP MIC (Table 1, row 7). The lack of an effect on CSP, SDS and CFW supports the argument that these changes in MIC for AMB, FLC and TRB are not simply growth effects related to the TET MIC.
The TET effect in other strains and species

In addition to the standard laboratory strain, four additional C. albicans isolates were tested, including two standard laboratory strains and two recent clinical isolates (Table 2). For these experiments, Etest strips were used instead of micro-dilution broth culture. The two techniques have been shown to give comparable MIC determinations (Pfaller et al., 1996). Using Etests, the AMB MIC for all strains was shown to be reduced three- to fivefold (Table 2, rows 1–5). The difference between the TET/AMB effect in Table 1 (32-fold) and in Table 2 (three- to fivefold) is likely the testing method, as SC5314 was tested in both systems. This demonstrates that the TET effect is observed in analyses in both broth and agar. The size of the effect (32-fold versus three- to fivefold) may be related to differential degradation of TET in agar versus broth in part because it is unstable in light and O2. The effect of TET on AMB susceptibility was also tested in two distantly related fungi, A. fumigatus and C. neoformans (Table 2, rows 6–7), where the AMB MIC was reduced approximately threefold. Thus, the TET effect on susceptibility to AMB appears to be consistent and at a similar level for all fungi tested.

The TET effect on the FLC MIC in the five C. albicans isolates was highly variable. Regarding SC5314, the effect of

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**Table 1. TET alters susceptibility to antifungal drugs**

<table>
<thead>
<tr>
<th>Row</th>
<th>Strain*</th>
<th>Drug</th>
<th>MIC† in TET concentrations (µg ml⁻¹):</th>
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<td>A</td>
<td>TRB</td>
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</tr>
<tr>
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<td>A</td>
<td>DTT</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
<td>SDS</td>
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<td>ND</td>
</tr>
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<td>A</td>
<td>CFW</td>
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<td>ND</td>
</tr>
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<td>9</td>
<td>B</td>
<td>AMB</td>
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<td>0.06</td>
</tr>
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</table>

* A, strain SC5314 (Fonzi & Irwin, 1993); B, strain DSY1050 (Mukherjee et al., 2003).
† MIC is 80% growth inhibition at 48 h as determined by CLSI protocol. ND, Not determined.
‡ Fold change=MIC₈₀ without TET compared to MIC₈₀ with 300 µg TET ml⁻¹. Negative signs indicate an increase in susceptibility. Positive signs indicate a decrease in susceptibility.
§ CSP, caspofungin. MICs were determined by Etests (see Methods).
|| DOX, doxycycline was used in place of TET.

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**Table 2. Changes in MIC with TET for various fungal species and strains**

<table>
<thead>
<tr>
<th>Row</th>
<th>Species*</th>
<th>Strain†</th>
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<th>AMB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td>+TET</td>
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<td>0.25</td>
</tr>
<tr>
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<tr>
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<td>3</td>
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<tr>
<td>7</td>
<td>Cn</td>
<td>H99</td>
<td>8</td>
<td>84</td>
</tr>
</tbody>
</table>

* Ca, Candida albicans; Af, Aspergillus fumigatus; Cn, Cryptococcus neoformans.
† References for strains: SC5314 (Fonzi & Irwin, 1993), 90028 (Espinell-Ingroff et al., 1992), 3153A (Evans et al., 1974), 2-76 (White et al., 1997), FH1 (Marc et al., 1997), AF293 (Nierz et al., 2005), H99 (Franzot et al., 1999) and D665-1A (Alarco et al., 2004).
‡ MICs were determined by Etests (see Methods).
§ NA, not applicable; FLC does not have activity against Aspergillus fumigatus.
TET on the FLC MIC was positive in broth culture (Table 1, row 1) and negative using Etests (Table 2, row 1). Variability was also seen with the other C. albicans clinical isolates (Table 2), where the effect of TET on the FLC MIC as determined by Etest was either positive or negative and did not differ by a factor of more than 2. Thus, there does not appear to be a consistent effect of TET on FLC susceptibility in C. albicans. The effect of TET on FLC susceptibility in A. fumigatus can not be determined as Aspergillus species are known to be intrinsically FLC resistant. However, the effect of TET on FLC susceptibility was highly significant in Cr. neoformans, increasing the FLC MIC 10-fold.

The TET effect is independent of efflux pumps

DSY1050 is a derivative of SC5314 that lacks the three major efflux pump genes associated with resistance to azoles, CDR1, CDR2 and MDR1. This strain was tested for the TET effect on the AMB MIC using broth (Table 1, row 8). If TET alters susceptibility through upregulation of any of these pump genes, then the triple deletion strain will not be altered by TET exposure. DSY1050 exposed to TET (Table 1, row 8) shows the same change in susceptibility as SC5314, suggesting that the TET effect on AMB susceptibility is independent of pump expression.

To further investigate the role of efflux in the TET effect, cells were tested with R123 and R6G, two fluorescent dyes and surrogate markers for drug accumulation that monitor efflux pump activity. R123 has been shown to compete with FLC in drug accumulation studies (Clark et al., 1996). In Candida, as in many other systems, R6G appears to be a substrate for efflux pumps including CDR1, CDR2 and MDR1 (Bouchara et al., 2000; Maesaki et al., 1999). No change was observed in R6G or R123 accumulation as determined by FACS when cells were grown in the presence of 200 μg TET ml⁻¹, compared to cells grown in the absence of drug (data not shown).

Luciferase analysis of gene expression

To elucidate the mechanism that results in altered susceptibility in the presence of TET, luciferase assays were conducted with strains of C. albicans in which the full-length promoters were fused to the luciferase gene, R-LUC (Harry et al., 2005; Song et al., 2004). Promoters tested included the efflux pump MDR1 (Harry et al., 2005), the azole target and sterol biosynthesis gene ERG11 (Song et al., 2004), and the transcription factor UPC2 (Silver et al., 2004) that regulates sterol biosynthesis gene expression. Promoters were tested in the absence and presence of 200 μg TET ml⁻¹ at 5 h, 24 h and 48 h. No significant changes were observed (not greater than a factor of 2) for any of the three genes. The MDR1 results are consistent with the MIC results using DSY1050 in suggesting that MDR1 expression is not related to the TET effect.

QRT-PCR analysis of gene expression

To further monitor gene expression, QRT-PCR was used to monitor expression of a collection of 27 genes associated with resistance and two control genes (ACT1 and CEF3) (Table 3). QRT-PCR was performed on RNA from cells grown with and without 200 μg TET ml⁻¹ in three biological replicates. The results were normalized to ACT1, with a focus on genes that are overexpressed or underexpressed at least twofold. The results were similar when the expression patterns were normalized to CEF3 instead of ACT1.

Four genes did show a fourfold increase in expression – two late-stage ERG genes, ERG2 and ERG6, and two efflux pumps, PDR16 and FLU1. While increased expression of these genes was reproducible, the increases were not statistically significant. In addition, the transcription factor CAP1 showed some increased expression but the results were variable, consistent with the low-level expression of transcription factors. The overexpression of genes encoding pumps and sterol biosynthetic enzymes is consistent with changes in MIC described above, which might be facilitated by increased drug efflux despite the data described above, or by changes in the later part of the ergosterol pathway. In addition, overexpression of CAP1 may contribute to pump regulation in response to TET, as CAP1 is activated in response to many hydrophobic drugs (Harry et al., 2005) and thus may be activated by TET. No change (average 1.33-fold reduced) was observed for UPC2, a transcription factor that regulates genes in the ergosterol pathway (Silver et al., 2004).

TET alters sterol levels

AMB binds to ergosterol (the fungal equivalent to mammalian cholesterol) and lyases cell membranes. For this reason any alteration in the level of ergosterol in the fungal membrane may alter the efficacy of AMB, perhaps through an increase in AMB target leading to increased susceptibility. Ergosterol scans are a technique in which cell membranes are extracted and the levels of ergosterol and other sterols are determined spectrophotometrically. These scans were performed to identify potential changes in ergosterol levels in the presence of TET (Fig. 2). Ergosterol levels were determined for SC5314 in the absence and presence of 200 μg TET ml⁻¹ at 24 and 48 h, which is the time at which relative growth is determined for MIC₈₀. No change was observed at 24 h in ergosterol levels (data not shown). A reproducible decrease in ergosterol (10–20% reduction) was observed at 48 h in the presence of TET (Fig. 2). This is consistent with the increase in MIC₈₀ for SC5314 exposed to FLC and TRB in the presence of TET and the decrease in MIC₈₀ when SC5314 is exposed to AMB in the presence of TET or DOX (less target to bind).

Effect of TET on growth

The growth of SC5314 was monitored for 7 days in the presence of 20 and 200 μg TET ml⁻¹ and in the presence of
vehicle (ethanol) alone. Growth was monitored by OD<sub>600</sub>. As can be seen in Fig. 3, cells cultured with and without TET grew at the same initial rate for at least 12 h. However, a change in growth rate was observed starting at 12 h. More important, the cells grown in the presence of TET reached a final cell concentration that is significantly below the final cell concentrations of cells grown with vehicle alone or with 20 mg TET ml<sup>−1</sup>. This difference in growth rate after 12 h or the difference in final cell concentration is unlikely to directly affect MIC determinations in either broth or agar, as MIC is usually measured at 80% growth at 48 h.

However, the change in growth pattern between 12 and 48 h is an important point. At approximately 24 h, fungal cells shift from fermentative growth utilizing glucose to respiratory growth utilizing the mitochondria, a change known as the diauxic shift. The shift is seen clearly in the

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**Table 3.** Primer sets used in QRT-PCR for genes associated with resistance

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<th>Gene*</th>
<th>Forward primer†</th>
<th>Reverse primer†</th>
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*All gene descriptions can be found in Arnaud et al. (2005).
†All primers are listed 5’ to 3’. Forward primer sequences match the coding strand; reverse primers are the reverse complement of the coding strand.

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**Fig. 2.** Ergosterol scans in cells exposed to TET. Ergosterol levels were analysed for strain SC5314 grown in the absence of TET (thin line) and the presence of 200 μg TET ml<sup>−1</sup> (heavy line). 300 OD<sub>600</sub> units of cells were used in each measurement.
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**Fig. 3.** Growth curve of strain SC5314 in the absence and presence of TET. Grey squares, cells grown with vehicle (ethanol) alone; open diamonds, cells grown with 20 μg TET ml⁻¹; open circles, cells grown with 200 μg TET ml⁻¹. Measurements are the mean of three separate time courses performed simultaneously. Standard deviations were smaller than the symbols and are not shown. Small variations at different times are probably due to variations in the spectrophotometer used in the analyses. The inset represents an enlargement of the data from 10 to 75 h, which shows the diauxic shift.

inset to Fig. 3, where cells grown with vehicle alone or with 20 μg TET ml⁻¹ grew from an OD₆₀₀ of about 5 to about 9. However, cells grown with 200 μg TET ml⁻¹ did not exhibit this growth, suggesting that cells grown under these conditions are unable to undergo the diauxic shift. This is consistent with the fact that TET affects mitochondrial as well as bacterial protein synthesis (Chopra & Roberts, 2001).

The link between mitochondria and TET was further explored by growing cells on media containing glycerol rather than glucose, since cells grown on glycerol can not undergo glycolysis and must rely on oxidative phosphorylation in the mitochondria. Using these conditions, the TET MIC is reduced and cells do not grow at 200 μg TET ml⁻¹. At lower concentrations (20 μg TET ml⁻¹), there is no detectable effect of TET on the MIC of FLC or AMB (data not shown).

**Mitochondrial function**

To document the effect of TET on mitochondrial function, the mitochondrial marker MTR was tested using fluorescence microscopy and FACS. As a control, mitochondrial function was inhibited with NaN₃. The microscopy results are shown in Fig. 4. Cells in the no-drug control show standard patterns of mitochondrial function, with ribbon-like mitochondria in most cells. Cells in the NaN₃ control show almost undetectable MTR staining. Cells in the presence of TET show a more diffuse staining with MTR and less intense staining of the mitochondria, indicating that mitochondrial function is reduced but not eliminated. To quantify these results, MTR-stained cells were analysed by FACS. When no drug was present, the geometric mean (arbitrary units, averaged from 10^4 cells) was 41.57; when no dye was present, the mean was 0.26. The geometric means were significantly reduced in the presence of 200 μg TET ml⁻¹ (7.96) to levels similar to the mean in the presence of 600 μM NaN₃ (5.10). FACS analysis of cells from both the TET and no-drug conditions resulted in a trace with two peaks, with the less intense peak containing fewer cells. This may be the result of the cell cycle, either exclusion of dye or reduced mitochondrial function during parts of the cell cycle. The double peak was not observed in the azide control.

**DISCUSSION**

This study investigated the effects of TET on the response to antifungals in *C. albicans* and other fungi. With the increased use of TET in inducible promoter systems, and with the previously reported altered susceptibility to AMB (Graybill & Mitchell, 1980; Raab & Hogl, 1980; Siau & Kerridge, 1998), it is important to understand the effects of TET on a cell, and to distinguish TET-related effects from gene expression phenotypes.

Susceptibility testing in the presence and absence of TET was performed using both the standardized microbroth dilution assays as defined by CLSI (Table 1 and Fig. 1) and Etest strips (Table 2), which normally correlate well with the standardized methods (Pfaller et al., 1996). The fact that both systems display the TET effect is consistent with the known correlation between the two systems. The levels of the effect do differ between the two methods; this is most likely the effect of growth in broth versus growth at an agar/air interface that may be related to differences in TET degradation and/or aeration.

The effect of TET on MIC is most significant with AMB and DOX (Fig. 1, Tables 1 and 2). The fact that both drugs exhibit the same effect, and that the effect is concentration dependent, suggests that the effect is directly related to the drugs and not a non-specific phenomenon. The effect of TET or DOX on AMB susceptibility is not observed below 50 μg ml⁻¹ (data not shown). To induce the TET promoter, some studies use 2–20 μg TET ml⁻¹ while other studies use 100–200 μg TET ml⁻¹ (Nakayama et al., 1998; Park & Morschhauser, 2005; Saville et al., 2003; Vogt et al., 2005). The studies using less than 50 μg ml⁻¹ of TET or DOX may not be directly affected by alterations in AMB susceptibilities. However, there may be many other changes in cellular phenotype at lower levels of TET or DOX.

The TET effect is observed with two other clinically important fungi – *Cr. neoformans* and *A. fumigatus* (Table 2, rows 6 and 7). The interactions between TET and AMB for these species are similar in direction and levels to the effect in *C. albicans*. The TET MIC for *Saccharomyces cerevisiae* is significantly below the TET MIC for *C. albicans* (data not shown). Therefore, the TET effect...
on antifungals such as AMB and FLC can not be tested with S. cerevisiae using the same TET levels as those used in this study.

TET can either increase or decrease the FLC MIC, depending on the strain used (Tables 1 and 2). The reasons for this variation are not clear, but the variations are not large in either direction. Odds et al. (1986) previously demonstrated altered MICs to FLC in the presence of TET. The previous experiments were performed using a non-standard MIC test, as the study pre-dates the CLSI protocol. In addition, the experiments were performed at a different temperature with rich, undefined media containing serum, which is important as FLC has considerable protein-binding abilities (Schafer-Korting et al., 1995).

TET does increase the TRB MIC substantially (32-fold, Table 1), which may correlate with the alteration in ergosterol levels that occurs in the presence of TET (Fig. 2) and may be related to the lack of a diauxic shift (Fig. 3). The increase in AMB susceptibility, the decrease in TRB susceptibility, the variable effects on FLC susceptibilities, and the corresponding decrease in ergosterol levels (Figs 1 and 2, Tables 1 and 2) all suggest that TET is having an effect (either direct or indirect) on sterol metabolism.

Susceptibility to DTT, a reducing agent with known effects on the cell wall, is dramatically decreased in the presence of TET (Table 1, row 4). The reduced susceptibility to DTT suggests that the influence of TET on mitochondrial function, oxidative state or ergosterol content protects cells from the effect of the reducing agent DTT on the cell wall and membrane.

To understand the effect of TET on antifungal drug susceptibility, analyses focused on known mechanisms of drug resistance. Luciferase assays and QRT-PCR did not

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**Fig. 4. TET effect on mitochondrial staining.**

Cells were incubated with and without TET (200 \( \mu \text{g \text{ml}^{-1}} \)) for 6 h prior to mitochondrial staining with MTR. NaN\(_3\) (600 \( \mu \text{M} \)) was used as a positive control for inhibition of mitochondrial function. Cells were observed by fluorescence microscopy and photographed. DIC, differential interference contrast.

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![MitoTracker and DIC images](http://mic.sgmjournals.org)
identify significant changes in expression of resistance genes, although altered expression of two ERG genes and two efflux pump genes, FLU1 and PDR16, marginally associated with resistance, was observed. FACS analyses with R123 and R6G did not detect differences in efflux pump activity in the presence of TET. While R123 and R6G are known to be substrates for the CDR pumps, the pumps encoded by FLU1 and PDR16 are not known to have an effect on the dyes. Importantly, strain DSY1050, which is deleted for the three most important efflux pumps associated with azole resistance, exhibits the same TET effect on AMB susceptibility (Table 1, row 8), suggesting that pumps are not important in this phenomenon.

There is a decrease in ergosterol levels at 48 h in the presence of TET. This difference is not observed at 24 h (data not shown), which is consistent with the growth curve (Fig. 3) and previous work suggesting that sterol stores are depleted over time up to 48 h (Song et al., 2004). The difference in ergosterol levels at 48 h may be related to the lack of a diauxic shift, the lack of functional mitochondria, and the significant oxygen requirements in sterol biosynthesis (12 molecules of oxygen for one molecule of ergosterol) (Hughes et al., 2007).

The lack of a diauxic shift (Fig. 3) is likely to be related to TET inhibition of mitochondrial protein synthesis. The loss of mitochondrial function is clearly demonstrated using the MTR dye (Fig. 4). There are clear links between antifungal drug susceptibilities and mitochondrial function in other fungal species. At least three groups have shown that C. glabrata mutants in mitochondrial function have a FLC-resistant phenotype (Defontaine et al., 1999; Kaur et al., 2004; Sanglard et al., 2001). Petite mutants (mitochondria defects or loss of mitochondrial DNA) have been shown to be FLC resistant in C. glabrata (Bouchara et al., 2000) and S. cerevisiae (Kontoyiannis, 2000). Similarly, mitochondrial mutants have been shown to be hypersusceptible to AMB in S. cerevisiae (Polevoda et al., 2006) and in C. glabrata (Bouchara et al., 2000). The effects of mitochondrial mutations in C. albicans have not been described in detail, although some mitochondrial mutants in C. albicans do show FLC resistance but no change in AMB susceptibility (Cheng et al., 2007). A detailed study of the mechanism of azole resistance and AMB hypersusceptibility in C. glabrata petite mutants demonstrated that the mutants overexpressed CDR-like efflux pumps, showed increase in efflux as measured by R6G, and were defective in sterol esterification (Brun et al., 2004). This study is in contrast to the results presented here, where efflux was not affected by TET inhibition of mitochondrial function.

A model for the TET effect as seen in these observations is as follows. TET inhibits mitochondrial function (Fig. 4), which eliminates the diauxic shift (Fig. 3). Lack of diauxic shift, or the lack of functional mitochondria alters sterol metabolism, resulting in lower ergosterol levels, consistent with the need for 12 molecules of oxygen to synthesize one molecule of ergosterol (Hughes et al., 2007). Lower sterol levels in cells grown in the presence of TET increase AMB susceptibility because of the higher AMB to ergosterol ratios at the cell surface. Changes in later stages of sterol biosynthesis result in a decrease in TRB susceptibility, and alterations in FLC susceptibility. However, altered sterol metabolism does not allow the full synthesis of ergosterol, again correlating with requirement for high levels of oxygen.

The use of TET as an inducible promoter remains a scientifically valid and useful tool. However, it is important to keep in mind that TET does have an effect on mitochondrial function, diauxic shift, sterol and carbohydrate metabolism, and antifungal susceptibilities. Studies in which TET alters the expression of a target gene must include controls for these processes. Another consideration is the use of strains with TET-inducible promoters in animal models. The potential for antagonism between TET and TRB, and for synergy between TET (or DOX) and AMB, should be considered when effective-dose drug concentrations are considered.

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