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

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Summary

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 to 200 µg/ml) 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 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 in 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 that TET has a direct effect on 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.

Keywords

Candida albicans; tetracycline; drug resistance; MIC; amphotericin B

Introduction

Tetracycline (TET), an antibiotic product of *Streptomyces* bacterium species, has been in use since the 1950's (Chopra & Roberts, 2001). The mechanism of action for TET and its

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derivative 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 effect 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), *Giardia 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 *C. albicans* (Park & Morschhauser, 2005), *Candida glabrata* (Nakayama *et al.*, 1998), *Schizosaccharomyces pombe* (Faryar & Gatz, 1992) and *A. 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 lab 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 1970's and 1980's, 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 & Hogg, 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.

Material and Methods

Abbreviations

AMB = amphotericin B, CFW = calcofluor white, CLSI - Clinical Laboratory Standards Institute, CSP = caspofungin, DOX = doxycycline, DTT - Dithiothreitol, FACS = fluorescence activated cell sorting, FLC = fluconazole, ITC = itraconazole, MIC - minimum inhibitory concentration, MTR = MitoTracker Red CMXRos, QRT-PCR - quantitative real time reverse transcription polymerase chain reaction, R123 = rhodamine 123, R6G = rhodamine 6G, R-LUC = Renilla reniformis luciferase gene, SDS - sodium dodecyl sulfate, TET = tetracycline, TRB = terbinafine,

Strains

The *C. albicans* strains used in this study were obtained as follows: SC5314 (W. Fonzi, Georgetown U.), DSY1050 (D. Sanglard, U. Hospital Lausanne), 90028 and 3153A (ATCC, Manassas, VA), and 2-76 and FH1 (our collection). Strains from other fungal species include *A. fumigatus* strain Af293 (Kieren Marr, Fred Hutchinson Cancer Research Center) and *C. neoformans* strain H99 (Jennifer Lodge, St. Louis U.). 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 that determines the minimal inhibitory concentration (MIC) of drug needed to inhibit cell growth (National Committee for Clinical Laboratory Standards, 2002). Cell growth was measured using a Beckman DU 640B spectrophotometer at 600 nm (Beckman Coulter, Fullerton, CA). MICs are reported using 20% relative growth (MIC₈₀) at 48 h (h). Isolates were grown in a 96 well plate containing a gradient of drug, and in four concentrations of TET (300 µg/ml, 200 µg/ml, 100 µg/ml, and 50 µg/ml). Etests were performed per manufacturer's directions (AB Biodisk, N.J.) 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* (Hoot & White, 2007) were grown in the presence or absence of either 20 µg/ml or 200 µg/ml of TET 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 the protocol described previously (Arthington Skaggs *et al.*, 1999). Strains were grown for 48 h, and 300 ODs of cells at 600 nm [concentration of cells (OD/ml) $\times$ volume (ml) = 300 OD] 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 0.2 OD and grown for 48 h in YEPD + TET (0, 50, 100, 200, 300 μ g/ml) in the dark at 30° C with shaking. After washing, the optical density of each culture was adjusted to 0.1 OD. A one 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 μ m 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 analyzed by flow cytometry using a Beckman Coulter Epics XL-MCL 4-color cell analyzer. 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 (Qiagen, Valencia, CA). 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 cDNA were subsequently analyzed in quantitative PCR using a SYBR green mastermix in an ABI 7500 (Applied Biosystems, Foster City, CA) following the manufacturer's recommended protocols.

Mitotracker Analyses

Cells were grown overnight in YAD at 30° C with shaking at 180 rpm. Cells were diluted to an OD of 0.1 in 10 mL YAD, YAD plus 200 μ g/mL TET, and YAD plus 600 μ M sodium azide (NaN_3). TET and NaN_3 treated cells were maintained in drug until fixation. After dilution, cells were incubated for 6 hours at 30° C, 180 rpm shaking in the dark. Cells were collected by centrifugation, and resuspended in fresh media with the mitochondrion-specific dye MitoTracker Red CMXRos (MTR, Invitrogen) at a final concentration of 20 nM. Cells were incubated for 15 min at 30° C, 180 rpm shaking 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 m at 30° C, 180 rpm in the dark. Stained fixed cells were washed 3 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 analyzed using a Beckman Coulter Epics XL-MCL 4-color cell analyzer. 10,000 cells per sample were analyzed. The geometric mean of FL3 channel

fluorescence for each sample was calculated using FlowJo software 7.2.2 (Tree star, Ashland, Oregon).

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 down regulation of genes in the ergosterol biosynthesis pathway. Further investigation of this inhibition revealed a *C. albicans* susceptibility to TET with a minimum inhibitory concentration (MIC₈₀) of 400 µg/ml, 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 AMB, using the standard laboratory strain SC5314 (Fig. 1A). Relative growth of cells in AMB was reduced with increasing concentrations of TET. This effect was titratable, from 50 to 300 µg/ml, which are below the 400 µg/ml 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 vitro, DOX also results 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 MIC₈₀. The MIC₈₀ values for the data from Figs 1A and 1B are presented in Table 2 (rows 1 and 8). For both TET and DOX in combination with AMB, the MIC₈₀ was reduced 32-fold for TET or DOX concentrations of 300 µg/ml. 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 µg/ml) increases the SC5314 MIC₈₀ to FLC 4-fold (Table 2, row 2). Terbinafine (TRB) inhibits *ERG1*, a gene upstream of *ERG11* in the ergosterol biosynthesis pathway. TET (200 to 300 µg/ml) increases the MIC₈₀ 32 fold (Table 2, 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. Dithiothreitol (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 2, 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, sodium dodecyl sulfate (SDS) disrupts membranes and cell walls, and TET had no effect on SDS (Table 2, row 5). Calcofluor white (CFW) also

interferes with cell wall stability and function, and TET had no effect on CFW (Table 2, row 6). Finally, caspofungin (CSP) is a clinically useful antifungal that targets glucan synthase in the cell membrane and inhibits the synthesis of the β 1,3 glucans in the cell wall. There was no TET effect on CSP MIC (Table 2, row 7). The lack of an effect on CSP, SDS and CFW support 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 is common to many fungi

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 3). 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 3 to 5 fold (Table 3, rows 1–5). The difference between the TET/AMB effect in Table 2 (32 fold) and in Table 3 (3 – 5 fold) 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 3–5 fold) 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 was also tested in two distantly related fungi, *A. fumigatus* and *C. neoformans* (Table 3, rows 6–7) where the AMB MIC was reduced approximately 3 fold. Thus, the TET effect on AMB appears to be consistent and at a similar level for all fungi tested.

The TET effect on FLC MIC in the five *C. albicans* isolates is highly variable. Regarding SC5314, the effect of TET on FLC MIC is positive in broth culture (Table 2, row 1), and negative using Etests (Table 3, row 1). Variability is also seen with the other *C. albicans* clinical isolates (Table 3) where the effect of TET on FLC MIC as determined by Etest is either positive or negative and does not differ by a factor of more than 2. Thus, there does not appear to be a consistent effect of TET on FLC in *C. albicans*. The effect of TET on FLC in *A. fumigatus* can not be determined as *Aspergillus* species are known to be intrinsically FLC resistant. However, the effect of TET on FLC is highly significant in *C. neoformans*, where TET increases 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 AMB MIC using broth (Table 2, rows 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 2, 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 Rhodamine 123 (R123) and Rhodamine 6G (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/ml TET, 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 include 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/ml TET at 5 h, 24h and 48h. 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, quantitative real time reverse transcription PCR (QRT-PCR) was used to monitor expression of a collection of 27 genes associated with resistance and two control genes (*ACT1*, and *CEF3*) (Table 1). QRT-PCR was performed on RNA from cells grown with and without 200 µg/ml TET in three biological replicates. The results were normalized to *ACT1*, with a focus on genes that are overexpressed or underexpressed at least two fold. The results were similar when the expression patterns were normalized to *CEF3* instead of *ACT1*.

Four genes did show a four-fold 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, they 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 lyses 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/ml TET at 24 and 48 h, which is the time at which relative growth is determined for MIC₈₀. No change was observed at 24 hours in ergosterol levels (data not shown). A reproducible decrease in ergosterol (10 to 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).

The TET effect on growth

The growth of SC5314 was monitored for 7 days in the presence of 20 µg/ml and 200 µg/ml of TET and in the presence of vehicle (ethanol) alone. Growth was monitored by optical density (OD). As can be seen in Fig 3, cells grown with and without TET grow at the same initial rate for at least 12 h. However, a change in growth rate is observed starting at 12 h. More important, the cells grown in the presence of TET reach a final cell concentration that is significantly below the final cell concentrations of cells grown in vehicle alone or in 20 µg/ml TET. This difference in growth rate after 12 h or the difference in final cell concentration is unlikely to directly affect MIC determinations either in 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 insert to Fig 2, where cells grown in vehicle alone or in 20 µg/ml TET grow from about 5 OD to 9 OD. However, in cells grown in 200 µg/ml TET, the cells do not exhibit this growth and remain at the same concentration. This suggests that cells grown in 200 µg/ml TET are not able to undergo the diauxic shift. This is consistent with the fact that TET is known to effect 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/ml TET. At lower concentrations (20 µg/ml TET), there is no detectable effect of TET on FLC or AMB MIC (data not shown).

Mitochondrial Function

To document the effect of TET on mitochondrial function, the mitochondrial marker MitoTracker Red CMXRos (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 a 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 quantitate these results, MTR stained cells were analyzed by FACS. The geometric means are presented in Table 4. The means were significantly reduced in the presence of TET to levels similar to the mean in the presence of azide.

Discussion

This study investigated the effects of TET on antifungal response 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 & Hogg, 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 2 and Fig 1) and Etest strips (Table 3) that normally correlate well with the standardized methods (Pfaffer *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, and 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 2 and 3). 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 TET or DOX effect on AMB susceptibility is not observed below 50 µg/ml (data not shown). To induce the TET promoter, some studies use 2 to 20 µg/ml while other studies use 100 to 200 µg/ml TET (Nakayama *et al.*, 1998; Park & Morschhauser, 2005; Saville *et al.*, 2003; Vogt *et al.*, 2005). The studies using less than 50 µg/ml 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 - *C. neoformans* and *A. fumigatus* (Table 3, 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 *S. 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 2 and 3). The reasons for this variation are not clear, but the variations are not large in direction. Odds *et al.* previously demonstrated altered MICs to FLC in the presence of TET (Odds *et al.*, 1986). 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 2), which may correlate with the TET alteration in ergosterol levels (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 2 and 3), 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 2, row 4). The reduced susceptibility to DTT suggests that the TET effect on mitochondrial function, oxidative state, or ergosterol content has a protective effect from the effect of DTT as a reducing agent with effects on the cell wall and membrane.

To understand the effect of TET on antifungal drug susceptibility, analyses were focused known mechanisms of drug resistance. Luciferase assays and QRT-PCR did not identify significant changes in expression of resistance genes, although altered expression of two *ERG* genes and two efflux pumps *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 (Table 2, 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 O₂ requirements in sterol biosynthesis (12 molecules of O₂ / 1 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 and Table 4). There are clear links between antifungal drug susceptibilities and mitochondrial function in other fungal species. At least two groups have shown that *C. glabrata* mutants in mitochondrial function have a FLC resistant phenotype (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 *Saccharomyces cerevisiae* (Kontoyiannis, 2000). Similarly, mitochondrial mutants have been shown to be hyper-susceptible 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 rhodamine 6G, and were defective in sterol esterification

(Brun *et al.*, 2004). This study is in contrast to the results presented here where efflux is 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 a functional mitochondria alters sterol metabolism resulting in lower ergosterol levels, consistent with the need for 12 molecules of O₂ to synthesis one molecule of ergosterol (Hughes *et al.*, 2007). Lower sterol levels in the cell 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 O₂.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1a.

Susceptibility testing of SC5314 with AMB in the presence of TET. SC5314 was tested using CLSI protocols for 48 h. Filled squares represent no TET, triangles represent 50 μg/ml of TET, inverted triangles represent 100 μg/ml TET, circles represent 200 μg/ml TET, and open squares represent 300 μg/ml TET.

Figure 1b. Susceptibility testing of SC5314 with AMB in the presence of DOX. SC5314 was tested using CLSI protocols for 48 h. Filled squares represent the no DOX control,

triangles represent 50 µg/ml DOX, inverted triangles represent 100 µg/ml DOX, circles represent 200 µg/ml DOX, and open squares represent 300 µg/ml DOX.

Figure 2. Ergosterol scans in cells exposed to TET. Ergosterol levels were analyzed for SC5314 grown in the absence (thin line) and the presence of 200 µg/ml of TET (heavy line). 300 OD of cells were used in each measurement.

Figure 3.

Growth curve of cells in the absence and presence of TET. Cell growth was monitored by O.D. (y axis) for 7 days (x-axis). Grey squares - cells grown in vehicle (ethanol) alone; open diamonds - cells grown in 20 µg/ml TET; open circles - cells grown in 200 µg/ml TET. Measurements are the average of 3 separate time courses performed simultaneously. Standard deviations are small and if graphed would be less than the symbols used. Small variations at different times are likely variations in the spectrophotometer used in the analyses. **Insert.** The insert represents an enlargement of the data from 10 to 75 h, which shows the diauxic shift.

Figure 4.

TET effect on mitochondrial staining. Cells were incubated with and without TET (200 $\mu\text{g/ml}$) for 6 hours prior to mitochondrial staining with MTR. Sodium azide (NaN_3 , 600 μM) was used as a positive control for inhibition of mitochondrial function. Cells were observed by fluorescence microscopy and photographed.

Table 1

Primer sets used in QRT-PCR for Genes associated with resistance

Gene ^a	Forward Primer ^b	Reverse Primer ^c
<i>ACT1</i>	acgggtgaagttgctgcttagtt	cgctgcaccggcaaaa
<i>ARE2</i>	cttcaggaaagtatatagctttcacaa	tgatgatcgctcgggattctct
<i>CAP1</i>	ttcgatagcaagaatggttcc	ggatcaccaaatgccaatgc
<i>CDR1</i>	aagatgctcgtcgcaagatgaatc	gagtgaagttctggctaaattctga
<i>CDR2</i>	ttgagccacatgtccgacat	ggaatctgggtctaattgttcata
<i>CDR3</i>	tgcacagcacaaattcatcac	tgctgtggtataggtatcatcgtaafg
<i>CDR4</i>	gcgcatcaagcacgtcaa	cgaacttgcataaaaaactaactg
<i>CEF3</i>	gctctcaagatggcaacttttc	gctgtcaagccatcttacaa
<i>CRZ1</i>	cttgctcagtttaccactaacga	gaaatcttgcagcagttgtgaataa
<i>ERG1</i>	gcagttgaagcaacagtaacaaaga	gggtgtttgtttgcacacaa
<i>ERG2</i>	ccgggtgaatcatttgccttag	tgaagtcaaagtatccaaaaatccaa
<i>ERG3</i>	cataaacctcatcacaaagtggattg	aaagattggaagaacccatcaact
<i>ERG5</i>	gctttgcagaccctgaag	accactagcattttcccatcttc
<i>ERG6</i>	catgactgataaatacagtaaaactaatga	actttacgagaatacattttggaatacc
<i>ERG9</i>	attctcattttgtgctattcctcaag	ctaattttgaccactccatgaaaactt
<i>ERG10</i>	gggtcatcctcttgggtgttct	ttaccaccttcttgaatcaagaca
<i>ERG11</i>	cccctattaattttgtttccctaattac	cacgttctcttctcagtttaattctttc
<i>FCR1</i>	aactgctgcaagattattgctgaa	gggttactagttggcattttgtgag
<i>FLU1</i>	tttatattgtgcatctgttggtcttg	gcacttgcctgagaaaaacattgc
<i>HMG1</i>	cagtattggcgcgtgaattatct	gcatatgtgattggaccaaataga
<i>HST6</i>	ctgacatcatcatcaacgtccttt	tgtgcattcgtcgaggagtaaat
<i>MDR1</i>	atcacccggaacgacagaatca	tctaattggctccataatgtatcaatga
<i>NCP1</i>	gaactggtgtgtcctccgttga	accaacattcacgccattctt
<i>NDT80</i>	tcaattgaatcaccgccttaaat	tgtagccctatacccagaagttctatc
<i>PDR16</i>	aaatcatctagtaccactagtttatccacagt	gggtctgaaggatgatgtgattcg
<i>RFG1</i>	aaccctgaagtttcccagaa	cagcaagattattccaatgttcctt
<i>SSN3</i>	gacattatacccagcagttgattt	tcgatttgcctttcacctt
<i>SSN8</i>	acattccatatcaatctcgatga	tgccttttaacccctgttca
<i>TAC1</i>	aagctgaagagaaattccctgatatt	agcaccaaccaatgcaatcat
<i>UPC2</i>	attatggattcgttagccaatgc	catgcagaagctggcaaaaga

^a All gene descriptions can be found at (Arnaud *et al.*, 2005).^b All primers are listed 5' to 3'. Forward primer sequences match the coding strand.^c All primers are listed 5' to 3'. Reverse primers are the reverse complement of the coding strand.

Table 2

TET alters susceptibility to antifungal drugs.

Row	Strain ^a	Drug	MIC ^b in TET concentrations (µg/ml)					Fold Change ^c
			0	50	100	200	300	
1	A	AMB	0.25	0.06	0.031	0.016	0.008	-32
2	A	FLC	0.13	0.25	0.25	0.5	0.5	+4
3	A	TRB	3.13	ND ^d	6.25	100	100	+32
4	A	DTT	2	ND	32	16	64	+32
5	A	SDS	0.013	ND	0.013	0.013	0.013	-
6	A	CFW	0.5	ND	0.5	0.5	0.5	-
7	A	CSP ^e	0.032	0.032	0.032	0.032	0.032	+4
8	A	AMB-DOX ^f	0.13	0.03	0.03	0.008	0.004	-32
9	B	AMB	0.25	0.06	0.03	0.004	0.004	-62.5

^aA = strain SC5314 (Fonzi & Irwin, 1993), B = strain DSY1050 (Mukherjee *et al.*, 2003).

^bMIC is 80% growth inhibition at 48 h as determined by CLSI protocol.

^cFold Change = MIC₈₀ without TET compared to MIC₈₀ in 300 µg/ml TET. Negative signs indicate an increase in susceptibility. Positive signs indicate a decrease in susceptibility.

^dNot Determined.

^eCSP = Caspofungin. MIC were determined using Etests which are commercially available.

^fDOX = Doxycycline was used in place of TET.

Table 3

Changes in MIC with TET for Fungal Species and Strains

Row	Species ^a	Strain ^b	FLC			AMB		
			MIC ^c		Fold Change	MIC ^c		Fold Change
			-TET	+TET		-TET	+TET	
1	Ca	SC5314	0.5	0.19	-2.6	0.032	0.012	-2.7
2	Ca	90028	0.5	1	2.0	0.064	0.023	-2.8
3	Ca	3153A	0.25	0.25	-	0.047	0.012	-3.9
4	Ca	2-76	0.25	0.5	2.0	0.047	0.016	-2.9
5	Ca	FH1	4	3	-1.3	0.032	0.006	-5.3
6	Af	AF293	NA ^d	NA	NA	0.25	0.094	-2.7
7	Cn	H99	8	84	10.5	0.125	0.047	-2.7

^aCa = *Candida albicans*, Af = *Aspergillus fumigatus*, Cn = *Cryptococcus neoformans*

^bReferences for strains: SC5314 (Fonzi & Irwin, 1993), 90028 (Espinel-Ingroff et al., 1992), 3153A (Evans et al., 1974), 2-76 (White et al., 1997), FH1 (Marr et al., 1997), AF293 (Nierman et al., 2005), H99 (Franzot et al., 1999), and D665-1A (Alarco et al., 2004)

^cMIC were determined by Etest.

^dNA = not applicable, FLC does not have activity against *Aspergillus fumigatus*.

Table 4

Geometric Means of FACS Analyses

Condition	Mean ^a
No drug	41.57 ^b
TET (200 µg/ml)	7.96 ^b
NaN ₃ (600 µM)	5.10
No dye	0.26

^a Geometric mean (arbitrary units) of 10,000 cells for the MitoTracker.

^b FACS analysis of both TET and no drug results 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.