Synergistic mechanism for tetrandrine on fluconazole against Candida albicans through the mitochondrial aerobic respiratory metabolism pathway

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We found that tetrandrine (TET) can reverse the resistance of Candida albicans to fluconazole (FLC) and that this interaction is associated with the inhibition of drug efflux pumps. Mitochondrial aerobic respiration, which plays a major role in C. albicans metabolism, is the primary source of ATP for cellular processes, including the activation of efflux pumps. However, it was unclear if TET exerts its synergistic action against C. albicans via its impact on the mitochondrial aerobic respiratory metabolism. To investigate this mechanism, we examined the impact of FLC in the presence or absence of TET on two C. albicans strains obtained from a single parental source (FLC-sensitive strain CA-1 and FLC-resistant strain CA-16). We analysed key measures of energy generation and conversion, including the activity of respiration chain complexes I and III (CI and CIII), ATP synthase (CV) activity, and the generation of reactive oxygen species (ROS), and studied intracellular ATP levels and the mitochondrial membrane potential (ΔΨm), which has a critical impact on energy transport. Mitochondrial morphology was observed by confocal microscopy. Our functional analyses revealed that, compared with strains treated only with FLC, TET + FLC increased the ATP levels and decreased ΔΨm in CA-1, but decreased ATP levels and increased ΔΨm in CA-16 (P<0.05). Additionally, CI, CIII and CV activity decreased by 23–48%. The production of ROS increased by two- to threefold and mitochondrial morphology was altered in both strains. Our data suggested that TET impacted mitochondrial aerobic respiratory metabolism by influencing the generation and transport of ATP, reducing the utilization of ATP, and resulting in the inhibition of drug efflux pump activity. This activity contributed to the synergistic action of TET on FLC against C. albicans.

INTRODUCTION

In the 1990s, the widespread use and abuse of the first-line drug fluconazole (FLC) led to treatment failure for the fungal pathogen Candida albicans, which had developed resistance to the drug (White et al., 1998) via several mechanisms, including reduced access of the drug because of the action of multidrug resistance efflux pumps (Odds, 1996; Hiller et al., 2006), mutation-mediated overexpression of genes that code for proteins targeted by azole drugs (Prasad & Kapoor, 2004) and the formation of the biomembrane (Mukhopadhyay et al., 2004). This resistance to FLC is responsible for the high morbidity and mortality among immunocompromised patients (Goffeau, 2008; Monk & Goffeau, 2008).

Tetrandrine (TET) is the major alkaloid component extracted from the plant Stephania tetrandra S. Moore, and has been used widely in the treatment of rheumatoid arthritis (Xu et al., 2008) and hepatic fibrosis (Feng et al., 2008). Our previous studies found that TET could reverse the resistance of C. albicans to FLC in vitro (Li & Zhang, 2006) and during the treatment of C. albicans-infected mice (Wang et al., 2007). The mechanism of action may be
related to the inhibition of the drug efflux system, and the expression of the drug efflux pump genes CDR1, CDR2, MDR1 and FLU1 (Zhang et al., 2009).

Mitochondrial aerobic respiratory metabolism plays a major role in C. albicans metabolism and the fermentation pathway plays a minor role (Zeuthen et al., 1988). The mitochondrion is the primary source of energy (ATP) for cellular growth, multiplication, apoptosis and other processes, such as drug efflux. To further explore the synergistic impact of TET and FLC on C. albicans, and the changes in mitochondrial aerobic respiration, we studied the FLC-sensitive C. albicans strain CA-1 and FLC-resistant strain CA-16 following exposure to FLC and/or TET. We examined key measures of energy production and conversion, including the activity of two respiration chain complexes [complexes I and III (CI and CIII)] and the ATP synthase (CV), and the generation of endogenous reactive oxygen species (ROS). Intracellular ATP levels and mitochondrial membrane potential (ΔΨm), which both have a critical impact on energy transport and the mitochondrial morphology, were also observed.

METHODS

Strains. The FLC-sensitive C. albicans strain CA-1 and FLC-resistant strain CA-16 (generously provided by Theodore C. White, University of Washington, USA) were confirmed previously to be derived from the same parental source (Pfaller et al., 1994; White et al., 1997). The responses of the strains were evaluated by the checkerboard microdilution method in the preliminary study according to the Clinical and Laboratory Standards Institute M27-A3 guidelines (CLSI, 2008). The MICs of FLC against the C. albicans strains CA-1 and CA-16 were 0.25 and 64 μg ml⁻¹, respectively, prior to treatment with TET; following treatment with TET (30 μg ml⁻¹), the FLC MICs for the two strains were 0.0625 and 8 μg ml⁻¹, respectively. The maximum non-cytotoxic concentration of TET (30 μg ml⁻¹) was consistent with the existing literature (Li & Zhang, 2006). For the following experiments, strains were grown in YPD (yeast extract peptone dextrose) at 2.5–5.0 × 10⁶ c.f.u. ml⁻¹ for 6 h at 37 °C, shaking at 200 r.p.m., in the presence or absence of FLC (750 μg ml⁻¹; Cipla, purity 99 %) and in the presence or absence of TET (30 μg ml⁻¹; Huico Plant, purity 99.6 %).

Real-time reverse transcription (RT)-PCR. C. albicans cultures incubated with or without drugs for 6 h were diluted to a concentration of 1.5 × 10⁷ c.f.u. ml⁻¹. Total RNA was extracted from the cells using the E.Z.N.A. Yeast RNA kit (Omega Bio-tek) according to the manufacturer’s protocol. cDNA was synthesized according to the manufacturer’s instructions (TakaRa, Biotechnology, Dalian, P. R. China). Real-time RT-PCR was performed using SYBR Green I to visualize and monitor the amplified product in a MiniOpticon Real-time PCR System (Bio-Rad). Gene-specific primers were designed as described previously (Park & Perlin, 2005; Yan et al., 2008). The PCR protocol (Wang et al., 2006) consisted of a denaturation step (95 °C for 10 s), and 40 cycles of amplification and quantification (95 °C for 10 s, 55 °C for 20 s and 72 °C for 15 s, with a single fluorescence measurement). The change in the fluorescence of the SYBR Green I dye in every cycle was monitored using the LightCycler system software (Roche Diagnostics) and the threshold cycle (Ct) above the background for each reaction was calculated. The Ct value of 18S rRNA was subtracted from that of the gene of interest to obtain a ΔCt value. The gene expression level relative to that of the calibrator was expressed as 2⁻ΔΔCt. Each strain in the absence of drug was used as the baseline control strain and the expression levels of the treated strains were quantified relative to that of the control strain.

Rh123 accumulation/efflux experiments. The percentage of Rh123-positive cells in C. albicans strains in the presence or absence of drugs was determined as described previously (Clark et al., 1996; Zhang et al., 2009). During the exponential growth phase, recovered C. albicans was suspended in 5 ml sorbitol (2.5 × 10⁶ c.f.u. ml⁻¹). Then 150 U lyticase and 125 μl β-mercaptoethanol (0.28 mol 1⁻¹) were added to the cells, and the suspension was incubated at 37 °C for 2 h. Cells were resuspended in RPMI 1640 (Gibco) broth and divided into five equal volumes (A–E) in separate test tubes following two washes with pre-cooled PBS buffer (4 °C). Rh123 was added to tubes A–D at a final concentration of 2 μg ml⁻¹. FLC, TET or TET + FLC was added to tubes B, C and D, respectively. Neither drugs nor Rh123 were added to tube E (control strain for background). For accumulation experiments, the tubes were then incubated at 37 °C for 45 min. For efflux experiments, the tubes were incubated at 37 °C for an additional 45 min. Samples were examined by flow cytometry (FACS Aria; BD Company) and 10,000 cells were counted in the FITC channel (λexcitation=488 nm, λemission=530 nm) to construct single-parameter histograms. Data were processed using Cellquest software and the percentage of Rh123-positive cells was calculated. Intracellular Rh123 fluorescence intensity (FI; λexcitation=488 nm, λemission=530 nm) before and after drug administration and after the accumulation/efflux experiments was measured at the same time.

Measurement of intracellular ATP levels. C. albicans strains incubated in the presence or absence of Antimycin A (AA; 2, 4 or 8 μg ml⁻¹; Enzo Life Sciences), FLC and/or TET were adjusted to a concentration of 1 × 10⁷ c.f.u. ml⁻¹. A total of 100 μl cell suspension was mixed with the same volume of BacTiter-Glo reagent (Promega) and incubated for 5 min at room temperature as described previously (Zhang et al., 2013). Luminescent signals were determined on a full wavelength multifunctional enzyme mark instrument (Siare®; Tecan). A control tube without cells was used to obtain a value for background luminescence. A standard curve of incremental ATP concentrations (from 1 μM to 10 μM) was constructed. Signals represented the mean of three separate experiments and the ATP content was calculated from the standard curve.

Assessment of ΔΨm. ΔΨm was measured using a Yeast Cell Mitochondria Membrane Potential Fluorescent Assay kit (GenMed Scientifics) as described previously (Misra et al., 2009). C. albicans strains (1 × 10⁷ c.f.u. ml⁻¹) incubated in the presence or absence of drugs for 6 h were stained with 10 μg ml⁻¹ JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide) at 30 °C for 20 min in the dark. Cells were washed twice, resuspended in PBS buffer, and measured on the full wavelength multifunctional enzyme mark instrument with an excitation wavelength of 490 nm and emission wavelength shifting from green (~530 nm) to red (~590 nm). ΔΨm was determined as a ratio of red to green FI.

Measurement of endogenous ROS production. ROS production was measured using a Yeast Cell Oxidative Stress Active Oxygen Fluorescence Assay kit (GenMed Scientifics) as described previously (Xiao et al., 2010). C. albicans strains (1 × 10⁷ c.f.u. ml⁻¹) incubated in the presence or absence of drugs for 6 h were stained with 10 μg ml⁻¹ CM-H₂DCFDA (6-chloromethyl- 2',7'-dichlorofluorescein diacetate, acetyl ester) at 30 °C for 20 min. Following staining, the cells were washed twice, resuspended in PBS buffer, and measured on the full wavelength multifunctional enzyme mark instrument with an excitation wavelength of 490 nm and emission wavelength shifting from 530 nm. ROS production was calculated by subtracting the FI value of cells in the absence of CM-H₂DCFDA from that of cells with CM-H₂DCFDA.
Isolation of mitochondria and assay for CI/CIII/CV activity. *C. albicans* incubated in the presence or absence of drugs for 6 h were washed and a total of $5 \times 10^6$ cells were prepared for each sample. Mitochondria were isolated using the Yeast Cell Mitochondria Isolation kit (GenMed Scientifics) as described previously (Xu et al., 2009). The isolated mitochondria were lysed using the Yeast Mitochondria Lysis kit (GenMed Scientifics) and the mitochondrial protein concentration was determined by the bichinchoninic acid method using a BCA Protein Concentration Quantification kit (GenMed Scientifics) (Bradford, 1976).

Complex activity was determined by colorimetric assay using the Fungus Mito Respiration Chain CI/CIII/CV Activity Assay kit (GenMed Scientifics) as described previously (Xu et al., 2009). Optical density was measured on an UV-visible spectrophotometer at wavelengths of 340–380/550/340 nm. One unit of CI/CIII/CV activity was defined as the amount of enzyme activity that oxygenated 1 mM NADH/CoQH$_2$/NADH min$^{-1}$ at 30 °C and pH 7.5.

Mitochondria morphology. *C. albicans* cells incubated in the presence or absence of drugs for 24 h were washed and a total of $1 \times 10^7$ cells were prepared for each sample. Mitochondria were stained with 1 mM MitoTracker Red (1:750) for 20 min in the dark. Results of Rh123 experiments as determined by flow cytometry. Compared with strains treated with FLC alone, the mean percentages of Rh123-positive cells (A1) increased significantly for both CA-1 and CA-16 strains treated with TET or TET + FLC following the accumulation experiments, but decreased significantly following treatment with TET or TET + FLC for the efflux experiments $(P<0.05)$. In the accumulation/efflux experiments, the mean percentages of Rh123-positive cells were not significantly different between TET and TET + FLC treatments $(P>0.05)$ (Table 2).

RESULTS

Effects of TET on the expression levels of genes and drug efflux pump activity

Relative expression levels of drug efflux pump genes in *C. albicans* strains. In both the FLC-sensitive strain CA-1 and FLC-resistant strain CA-16, mRNA levels for $CDR1$, $CDR2$, $MDR1$ and $FLU1$ all decreased $(P<0.05)$ upon treatment with TET + FLC compared with treatment with FLC alone. Additionally, TET + FLC treatment decreased the expression levels of $CDR1$, $CDR2$, $MDR1$ and $FLU1$ in both strains compared with treatment with TET $(P<0.05)$. These results suggested that TET + FLC could downregulate the expression of the energy-related drug resistance genes $CDR1$ and $CDR2$ (Table 1, Fig. 1).

<table>
<thead>
<tr>
<th>Gene</th>
<th>CA-1</th>
<th>TET</th>
<th>TET + FLC</th>
<th>CA-16</th>
<th>FLU1</th>
<th>TET</th>
<th>TET + FLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FLC</td>
<td>TET</td>
<td>TET + FLC</td>
<td></td>
<td></td>
<td></td>
<td>TET + FLC</td>
</tr>
<tr>
<td>$CDR1$</td>
<td>$1.19 \pm 0.01$</td>
<td>$2.16 \pm 0.13$</td>
<td>$1.08 \pm 0.03$</td>
<td>$0.65 \pm 0.02$</td>
<td>$0.53 \pm 0.06$</td>
<td>$0.32 \pm 0.01$</td>
<td></td>
</tr>
<tr>
<td>$CDR2$</td>
<td>$1.73 \pm 0.15$</td>
<td>$2.28 \pm 0.13$</td>
<td>$1.34 \pm 0.44$</td>
<td>$0.74 \pm 0.05$</td>
<td>$0.52 \pm 0.04$</td>
<td>$0.44 \pm 0.04$</td>
<td></td>
</tr>
<tr>
<td>$MDR1$</td>
<td>$1.29 \pm 0.06$</td>
<td>$4.29 \pm 0.16$</td>
<td>$1.16 \pm 0.06$</td>
<td>$1.76 \pm 0.14$</td>
<td>$1.49 \pm 0.08$</td>
<td>$1.44 \pm 0.04$</td>
<td></td>
</tr>
<tr>
<td>$FLU1$</td>
<td>$1.57 \pm 0.11$</td>
<td>$2.55 \pm 0.45$</td>
<td>$1.33 \pm 0.22$</td>
<td>$0.7 \pm 0.03$</td>
<td>$0.73 \pm 0.02$</td>
<td>$0.61 \pm 0.02$</td>
<td></td>
</tr>
</tbody>
</table>

Relative expression levels of genes in *C. albicans* strain CA-1/CA-16 treated with FLC (750 µg ml$^{-1}$) and/or TET (30 µg ml$^{-1}$) were quantified relative to the control strain (CA-1/CA-16 untreated with drug). Values represent mean $\pm$ SEM of three independent LightCycler reactions.

Effects of TET on *C. albicans* Rh123 accumulation and efflux

Results of Rh123 experiments as determined by flow cytometry. Compared with strains treated with FLC alone, the mean percentages of Rh123-positive cells (A1) increased significantly for both CA-1 and CA-16 strains treated with TET or TET + FLC following the accumulation experiments, but decreased significantly following treatment with TET or TET + FLC for the efflux experiments $(P<0.05)$. In the accumulation/efflux experiments, the mean percentages of Rh123-positive cells were not significantly different between TET and TET + FLC treatments $(P>0.05)$ (Table 2).

Effects of TET on mitochondrial energy conversion

TET can inhibit CI and CIII activity. Compared with FLC treatment, mitochondrial respiratory chain CI and CIII activity was inhibited upon treatment with TET + FLC for both CA-1 and CA-16 strains $(P<0.05)$; 40 % decrease in CI activity compared with FLC in CA-1 and 48 % decrease in CIII activity; 38 % decrease in CI activity compared with FLC in CA-16 and 45 % decrease in CIII activity). Compared with drug-free/FLC-treated cells, the enzymic activities of CI and CIII were decreased significantly for both strains upon treatment with TET + FLC $(P<0.05)$ (Table 3, Fig. 2a, b).
These results suggested that TET could decrease the production of ATP by inhibiting CI and CIII activity.

**TET can inhibit CV activity.** Compared with FLC-treated cells, CV activity was decreased significantly in both CA-1 and CA-16 treated with TET + FLC ($P<0.05$, 41% decrease compared with FLC-treated cells for CA-1 and 23% decrease for CA-16). Compared with drug-free/TET-treated cells, the enzymic activity of CV decreased significantly in both strains upon treatment with TET + FLC ($P<0.05$) (Table 3, Fig. 2c). These results demonstrated that TET could further decrease ATP levels by inhibiting CV activity.

**Combination of TET and FLC augments endogenous ROS production.** Compared with FLC-treated cells, the concentration of endogenous ROS in both CA-1 and CA-16 cells treated with TET + FLC increased significantly ($P<0.05$, threefold increase compared with FLC alone for CA-1 and twofold increase for CA-16). Compared with drug-free/TET-treated cells, TET + FLC treatment resulted in a significant increase in the production of endogenous ROS in both strains ($P<0.05$) (Fig. 3). These results indicated that the combination of TET and FLC could increase endogenous ROS in *C. albicans*.

**Effects of TET on mitochondrial energy transport**

TET can regulate the intracellular ATP content in *C. albicans*. Compared with drug-free cells, treatment with the suppressive agent AA (2, 4 or 8 μg ml$^{-1}$) caused a significant decrease in the intracellular ATP content of cells of both strains ($P<0.05$). However, the differences were

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**Table 2. Percentages of Rh123-positive cells and intracellular Rh123 FI values for *C. albicans* strains CA-1 and CA-16**

<table>
<thead>
<tr>
<th>Strain</th>
<th>A1 (%)</th>
<th>FI1</th>
<th>E1 (%)</th>
<th>FI2</th>
<th>E2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.1</td>
<td>203</td>
<td>0.1</td>
<td>189</td>
<td></td>
</tr>
<tr>
<td>CA-1</td>
<td>57.1</td>
<td>1531</td>
<td>12.4</td>
<td>482</td>
<td>78.46</td>
</tr>
<tr>
<td>CA-1 + FLC</td>
<td>60.8</td>
<td>1620</td>
<td>10.8</td>
<td>463</td>
<td>82.4</td>
</tr>
<tr>
<td>CA-1 + TET</td>
<td>76.6</td>
<td>3420</td>
<td>42.1</td>
<td>1595</td>
<td>45.17</td>
</tr>
<tr>
<td>CA-1 + TET + FLC</td>
<td>78.9</td>
<td>3665</td>
<td>48.9</td>
<td>1899</td>
<td>38.15</td>
</tr>
<tr>
<td>B2</td>
<td>0.2</td>
<td>107</td>
<td>0.2</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>CA-16</td>
<td>17.8</td>
<td>1258</td>
<td>5.5</td>
<td>383</td>
<td>70.22</td>
</tr>
<tr>
<td>CA-16 + FLC</td>
<td>23.1</td>
<td>1480</td>
<td>4</td>
<td>351</td>
<td>83.55</td>
</tr>
<tr>
<td>CA-16 + TET</td>
<td>47.1</td>
<td>2640</td>
<td>19.8</td>
<td>696</td>
<td>58.39</td>
</tr>
<tr>
<td>CA-16 + TET + FLC</td>
<td>47.6</td>
<td>2624</td>
<td>19.1</td>
<td>764</td>
<td>60.29</td>
</tr>
</tbody>
</table>

B1 and B2, background controls for CA-1 and CA-16, respectively. A1, percentage of Rh123-positive cells (background subtracted) after the accumulation experiment; E1, percentage of Rh123-positive cells (background subtracted) after the efflux experiment; E2, percentage of efflux Rh123-positive cells; FI1 and FI2, FIs after the accumulation and efflux experiments. FLC, 750 μg ml$^{-1}$; TET, 30 μg ml$^{-1}$.
not significant between the different AA concentrations for the same strain ($P>0.05$) (Fig. 4a).

Compared with FLC-treated cells, the intracellular ATP content of cells treated with TET + FLC increased significantly in CA-1 cells, but decreased in CA-16 cells ($P<0.05$). Compared with the drug-free cells, TET + FLC treatment increased the intracellular ATP content of CA-1 cells, but decreased the intracellular ATP content of CA-16 cells ($P<0.05$) (Fig. 4b). These results suggested that TET could alter the production of intracellular ATP in $C. albicans$.

**TET can regulate $\Delta \Psi_m$.** Compared with FLC-treated cells, TET + FLC treatment resulted in a significant decrease in $\Delta \Psi_m$ for CA-1, but a significant increase for CA-16 ($P<0.05$). Compared with drug-free cells, $\Delta \Psi_m$ increased significantly for FLC-treated cells, but decreased significantly for TET + FLC-treated cells for CA-1 ($P<0.05$). However, $\Delta \Psi_m$ did not change significantly for FLC-treated CA-16 cells ($P>0.05$), but was decreased significantly in TET + FLC-treated CA-16 cells ($P<0.05$) (Fig. 5). These results suggested that TET treatment could alter the efficiency of mitochondrial aerobic respiration by adjusting the $\Delta \Psi_m$.

### Effects of TET on mitochondrial morphology
A large number of mitochondria were observed in both CA-1 and CA-16 drug-free cells, and the mitochondria were granular and dispersed. However, mitochondrial morphology changed in both strains following treatment with drugs for 24 h. Compared with FLC-treated cells, both the strains treated with TET + FLC exhibited an extensive tubular network of mitochondria, which were elongated and fused (Fig. 6).

### DISCUSSION
TET is known to reverse FLC drug resistance in $C. albicans$ by inhibiting the function of drug efflux pumps and this activity is related to energy metabolism (Pfaller et al., 1994); however, the mechanism of this synergistic interaction of TET against FLC-resistant $C. albicans$ remained unknown. Mitochondrial aerobic respiratory metabolism, which consists of energy generation (liberation), conversion and utilization (consumption), plays a key role in $C. albicans$ energy metabolism (White et al., 1997; Kusch et al., 2008). The inhibitory activity of TET against drug efflux pump function in $C. albicans$ is related to one or more aspects of its influence on cellular mitochondrial energy generation, conversion and utilization or to its impact on the structure and activity of the efflux pump. This model serves as the foundation of our research design.

In this study, we demonstrated that compared with FLC-treated cells, TET + FLC treatment can influence the production and conversion of ATP and promote cellular apoptosis by significantly inhibiting the enzymic activity of the respiration chain complex (CI, CIII and CV) and increasing endogenous ROS production. TET + FLC

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**Table 3. Relative activity of mitochondrial respiratory chain CI/CIII/CV in $C. albicans$ strains CA-1 and CA-16**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CI</td>
</tr>
<tr>
<td>CA-1 + FLC</td>
<td>37.60</td>
</tr>
<tr>
<td>CA-1 + TET</td>
<td>30.09</td>
</tr>
<tr>
<td>CA-1 + TET + FLC</td>
<td>15.00</td>
</tr>
<tr>
<td>CA-16 + FLC</td>
<td>68.55</td>
</tr>
<tr>
<td>CA-16 + TET</td>
<td>54.51</td>
</tr>
<tr>
<td>CA-16 + TET + FLC</td>
<td>30.80</td>
</tr>
</tbody>
</table>

These results were compared with the results in the control strain (CA-1/CA-16 untreated with drug). FLC, 750 µg ml$^{-1}$; TET, 30 µg ml$^{-1}$.

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**Fig. 2.** (a) CI activity, (b) CIII activity and (c) CV activity in $C. albicans$ strains CA-1 and CA-16 untreated or treated with FLC (750 µg ml$^{-1}$) and/or TET (30 µg ml$^{-1}$). $^*P<0.05$ versus control for CA-1, $^{**}P<0.05$ versus CA-1 + TET + FLC, $^{*}P<0.05$ versus control for CA-16, $^{**}P<0.05$ versus CA-16 + TET + FLC.
treatment alters mitochondrial morphology and affects the level of ATP transport from the mitochondrial matrix to the cytoplasm, which results in a decrease in available ATP by regulating intracellular ATP content and $\Delta \Psi_m$. These biochemical processes may impinge on the mechanism of synergistic action between TET and FLC against C. albicans.

We sought to determine how TET affects mitochondrial energy generation and conversion in C. albicans. The classical respiratory chain (CRC) is the major source of respiratory activity and $O_2$ consumption in C. albicans. The CRC provides the largest amount of cellular ATP by transmitting electrons through CI, CIII and CIV; subsequently, the electrons and $O_2$ fuse together, releasing energy (Alonso-Monge et al., 2009; Li et al., 2011). In the CRC pathway, CI, CIII and CIV oxidoreductases on the inner mitochondrial membrane produce $H^+$ ions that are pumped into the intermembrane space (Li et al., 2011), generating an electrochemical proton gradient between the intramembrane space and outer membrane. $\Delta \Psi_m$ is then used by the inner mitochondrial membrane CV to synthesize ATP, which completes mitochondrial energy conversion. In this way, the CI, CIII and CV components of electron flow in the CRC are important targets for energy generation and conversion (Li et al., 2011).

We found that CI, CIII and CV activity was inhibited significantly in both FLC-sensitive CA-1 and FLC-resistant CA-16 cells treated with TET $+$ FLC (Fig. 2), suggesting that TET $+$ FLC can reduce the production and conversion of mitochondrial ATP by inhibiting the activities of these key complexes (CI, CIII and CV) in C. albicans. AA, a known inhibitor of CIII (Milani et al., 2001), can decrease significantly the cellular ATP content in both strains, and treatment with TET $+$ FLC can similarly decrease significantly cellular ATP content in the FLC-resistant strain CA-16. These results indicate that TET $+$ FLC has a similar

![Fig. 4. (a) Intracellular ATP content in C. albicans strains CA-1 and CA-16 untreated or treated with AA [2 (A2), 4 (A4) and 8 (A8) $\mu g \text{ml}^{-1}$, respectively]. *$P<0.01$ versus control for CA-1, **$P<0.01$ versus control for CA-16. (b) Intracellular ATP content in C. albicans strains CA-1 and CA-16 untreated or treated with FLC (750 $\mu g \text{ml}^{-1}$) and/or TET (30 $\mu g \text{ml}^{-1}$) for 6 h. *$P<0.01$ versus control for CA-1, **$P<0.01$ versus CA-1 $+$ TET $+$ FLC, #*$P<0.01$ versus control for CA-16, #*$P<0.01$ versus CA-16 $+$ TET $+$ FLC.](http://jmm.sgmjournals.org)
inhibitory action against CIII as an inhibitor, i.e. AA, and this inhibitory action maybe part of the synergistic mechanism of TET and FLC against FLC-resistant C. albicans.

Treatment with TET also alters the level of mitochondrial energy transport during its synergistic action against C. albicans. The transportation of ATP from the mitochondrial matrix to the cytoplasm is affected by the cell’s ΔΨm and ATP/ADP translocase (Fig. 7). This study demonstrated that the intracellular ATP content and level of ΔΨm changes dramatically in both strains treated with TET + FLC, although the changes were not identical (Fig. 5). The FLC-sensitive strain CA-1 treated with FLC or TET + FLC was in a stringent state. Thus, mitochondrial metabolism was feedback activated, resulting in the mitochondria releasing more ATP. However, the ΔΨm of CA-1 cells decreased and the transport of ATP to the cytoplasm was reduced, resulting in a reduction in the available energy. For FLC-resistant CA-16 cells, the ΔΨm increased and the production of ATP decreased upon treatment with TET + FLC. This reflects differences in energy metabolism between the two strains and may be correlated with the dose of FLC. These results suggest that treatment with TET + FLC may influence the transport of ATP and alter the efficiency of aerobic respiration by adjusting the ΔΨm. These changes may result in the synergistic activity of TET against C. albicans by reducing available ATP.

The synergistic effect of TET against C. albicans is associated with the production of endogenous ROS. ROS is the byproduct of electron transfer in mitochondria, and is produced primarily by CI and CIII. Excessive production and leakage of ROS disrupts the redox equilibrium of mitochondria, which can lead to oxidative stress in the cell. Excessive ROS was confirmed to be correlated closely with cellular aging, apoptosis and the selective degradation of mitochondria (Drakulic et al., 2005; Wilhelm et al., 2006; Soengas, 2012). Li et al. (2011) and Ruy et al. (2006) have demonstrated that oxidative stress and enhanced

![Fig. 5. ΔΨm in C. albicans strains CA-1 and CA-16 treated or untreated with FLC (750 μg ml⁻¹) and/or TET (30 μg ml⁻¹) for 6 h. *P<0.05 versus control for CA-1, **P<0.05 versus CA-1 + TET + FLC, ###P<0.05 versus control for CA-16, ##P<0.05 versus CA-16 + TET + FLC.](image)

![Fig. 6. Mitochondria were stained with MitoTracker Red and viewed by confocal microscopy: (a) CA-1, (b) CA-1 + FLC, (c) CA-1 + TET, (d) CA-1 + TET + FLC, (e) CA-16, (f) CA-16 + FLC, (g) CA-16 + TET, (h) CA-16 + TET + FLC. Bar, 5 μm.](image)
production of ROS occur following the disruption of CI and/or CIII or when CIII is partially inhibited, which leads to cellular apoptosis in \textit{C. albicans}. Our data also confirm that following combination treatment with TET + FLC, endogenous ROS production increases significantly in both CA-1 and CA-16 (Fig. 3). This suggests that TET can influence aerobic respiratory metabolism in mitochondria by increasing the production of endogenous ROS, leading to cellular apoptosis and exerting a synergistic effect on \textit{C. albicans}.

We also found that mitochondria morphology was altered after treatment of \textit{C. albicans} with TET. Under normal conditions, \textit{C. albicans} cells contain numerous mitochondria, which are dispersed as granules throughout the cytoplasm. After treatment with FLC/TET for 24 h, the mitochondria became elongated and fused, particularly following combined treatment with TET+FLC, and the mitochondria appeared as a tubular network structure (Fig. 6). This change in mitochondrial morphology is closely related to cellular energy metabolism, autophagy and cell death (Gomes et al., 2011). Under external pressure, the elongated, fused or tubular network of mitochondria can increase ATP synthesis to recover from damage. However, once the elongation of mitochondria is blocked, either due to heredity or pathological changes, ATP is consumed in an uncontrolled manner, leading to cellular death. We hypothesize that prolonged exposure to TET + FLC further alters mitochondrial morphology, and that this is accompanied by a decrease in the number of mitochondria and excessive consumption of ATP. This ultimately leads to autophagy and degradation of \textit{C. albicans} because of an insufficient energy supply.

Taken together, our data indicate that treatment with TET may inhibit the metabolic processes of mitochondrial energy conversion or transportation and decrease the synthesis or transport ATP within mitochondria, thus inhibiting the excretory activity of drug efflux pumps and increasing the production of endogenous ROS. Subsequently, the morphology of mitochondria is altered, which leads to apoptosis. In this manner, TET may exert synergistic activity with FLC against \textit{C. albicans}. However, the synergistic action of TET against \textit{C. albicans} is a complicated process with multiple factors and mechanisms. Therefore, we will investigate further whether there are alternate methods for TET to exert its synergistic action against \textit{C. albicans} and clarify their mechanisms.

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