Tea polyphenol epigallocatechin-3-gallate inhibits ergosterol synthesis by disturbing folic acid metabolism in *Candida albicans*

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**Objectives:** Elucidation of the mechanism of action of epigallocatechin-3-gallate (EGCG) against *Candida albicans* and demonstration of the connection between its antifolate activity and other metabolic pathways involved in *C. albicans* survival are the major objectives of this study.

**Methods:** *C. albicans* ATCC 10231 and 12 clinical isolates were used. MICs of EGCG against *C. albicans* were determined according to NCCLS. *C. albicans* dihydrofolate reductase (DHFR) was purified using methotrexate-affinity chromatography and its inhibition by EGCG studied by spectroscopic techniques. Synergy experiments were performed by chequerboard tests by combining eight doubling concentrations of EGCG with another eight dilutions ofazole compounds or terbinafine. Reversion experiments with leucovorin or S-adenosylmethionine were carried out, and the content of ergosterol was determined by a spectrophotometric method.

**Results:** EGCG is an efficient inhibitor of *C. albicans* DHFR ($K_i = 0.7 \mu M$). As with other antifolates, the effects of EGCG on *C. albicans* can be highly attenuated by growing the cells in the presence of leucovorin. EGCG showed synergy with inhibitors of the ergosterol biosynthesis pathway in *C. albicans* such as azole antifungals and terbinafine. We demonstrate that by disturbing the folic acid metabolism, EGCG can inhibit ergosterol production. The molecular connection between the pathways is discussed.

**Conclusions:** EGCG acts as an antifolate compound on *C. albicans*, disturbing its folic acid metabolism. This effect could explain the molecular mechanism for the synergy between EGCG and azole antifungals, and could represent a starting point for therapies involving antifolates and azoles used as an alternative for the treatment of *C. albicans* infections.

Keywords: dihydrofolate reductase; antifolates; candidosis

**Introduction**

The increased incidence of invasive mycoses and the emerging problem of antifungal drug resistance have encouraged the search for new antifungal agents. *Candida albicans* is the most common fungal pathogen in humans, where it accounts for a large proportion of all fungal diseases, and thus represents a serious public health challenge of increasing medical and socio-economic importance. *Candida* species are ubiquitous commensal yeasts that usually reside as part of an individual’s normal mucosal (oral cavity, gastrointestinal tract and vagina) microflora. However, if the balance of the normal flora is disrupted or the immune defences are compromised, *Candida* species can invade mucosal surfaces and cause disease. Infections due to *C. albicans* are usually treated with azole antifungals such as fluconazole and itraconazole. Azoles inhibit the enzyme sterol 14α-demethylase in the sterol biosynthetic pathway. This pathway is conserved in euukaryotes, leading to cholesterol in mammals and ergosterol in fungi. In *C. albicans* sterols have been shown to be important in membrane fluidity and permeability, cell morphology, enzyme activity and cell-cycle progression. While azoles have little or no toxicity, they generally offer rather poor fungicidal activity. Even in the absence of resistance, treatment failures or recurrent infections are not uncommon, especially in immunocompromised individuals. These clinical limitations associated with the use of azoles have made the search for novel antifungal drugs more necessary.

Recent studies have presented data on a number of biological activities of tea polyphenols or catechins. It is of importance to our study that tea catechins have been reported to have shown...
antifungal activity against *C. albicans*.13 However, the exact action mechanism(s) of tea catechins on *C. albicans* was (were) not well defined. Deciphering the molecular mechanism by which green tea or its major component (epigallocatechin-3-gallate; EGCG) imparts its antifungal effects is important because it could result in improved opportunities for the treatment of a variety of fungal infections. Recently, we have shown that ester-bonded gallate catechins isolated from green tea, such as EGCG and ECG, are potent inhibitors of dihydrofolate reductase (DHFR) activity in vitro.14–16 at concentrations found in the serum and tissues of green tea drinkers (0.1–1.0 μM).17 DHFR is a key enzyme in the biosynthesis of purines, pyrimidines and several amino acids,18 and is the target of a number of drugs, including the antimicrobial agent trimethoprim or the antitumour drug methotrexate. However, antifolates are not used for the treatment of *C. albicans* infections because trimethoprim is a very weak inhibitor of *C. albicans* DHFR,19 while methotrexate, which binds tightly to this DHFR,20 exhibits poor antifungal activity.21 The elucidation of the mechanism of action of EGCG against *C. albicans* and a demonstration of the connections between its antifolate activity and other metabolic pathways involved in *C. albicans* survival are the main objectives of this study.

Materials and methods

**Microorganisms and growth conditions**

*C. albicans* ATCC 10231 and 12 clinical isolates were used in this study. Clinical isolates were collected over a period of 6 months at the Hospital Universitario Virgen de la Arrixaca (Murcia, Spain). All strains were maintained routinely on Sabouraud dextrose agar (Oxoid Ltd, Basingstoke, UK). Incubation before susceptibility testing was performed aerobically on this medium at 35°C for 24–48 h.

**EGCG and antifungals**

EGCG was obtained from Sigma Chemical Co. (Madrid, Spain). Stock dilutions were prepared in 0.15 mM H₃PO₄ to avoid oxidation. Itraconazole and ketoconazole were also obtained from Sigma. Stock solutions (5 g/L) were prepared in DMSO and stored for a maximum of 2 months at −20°C until use. Terbinafine from Novartis (Barcelona, Spain) was dissolved in DMSO (12.8 g/L), diluted 2× in DMSO, and then diluted 50× in complete medium. Trimethoprim, methotrexate, pyrimethamine and 5-fluorocytosine were obtained from Sigma.

**Purification of C. albicans DHFR**

DHFR was purified from *C. albicans* strain ARX1 using, among other techniques, methotrexate-affinity chromatography.19 The strain was inoculated in BHI broth Bacto™ Brain Heart Infusion (Becton, Dickinson and Company, Le Pont de Claix, France) and incubated at 35°C with shaking at 100 cycles per min for 48 h. After this, yeasts were harvested by centrifugation (1600 rpm; 30 min) and washed twice in 50 mM phosphate buffer (pH 7.0) followed each time by a new centrifugation (1600 rpm; 5 min). Cell lysis, centrifugation and dialysis were carried out between 4 and 8°C. Fast protein liquid chromatography (FPLC) purification steps were performed at room temperature. Cell paste from 2 L of culture was suspended in 30 mL of buffer A (5 mM Tris·HCl, pH 7.4, 1 mM EDTA) containing a protease inhibitor cocktail (5 μM pepstatin A, 1.5 μM bestatin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mM 1,10-phenanthroline), and the cell suspension was subjected to homogenization in a Potter homogenizer followed by ultrasonication. After centrifugation at 36,000 rpm for 30 min to remove cell debris, the supernatant was filtered and used for further purification steps. The DHFR concentration was determined by MTX titration of enzyme activity,22 while the total protein concentration was determined using bovine serum albumin as standard and an acidic solution of Coomassie® Brilliant Blue G-250 dye (Bio-Rad protein assay).

**DHFR assay and kinetic data analysis**

DHFR was obtained from Aldrich Chemie GmbH (Madrid, Spain) and NADPH from Sigma. DHFR activity was determined at 25°C by following the decrease in absorbance of NADPH and DHF at 340 nm (ε = 11 800 M⁻¹cm⁻¹) using a Perkin-Elmer Lambda-35 spectrophotometer with 1.0 cm light-path cuvettes. Temperature was controlled at 25 ± 0.1°C using a Haake D1G circulating bath with a heater/cooler. Experiments were performed in a buffer containing 2-(N-morpholino)ethanesulfonic acid (Mes, 25 mM), sodium acetate (25 mM), tris(hydroxymethyl)aminomethane (Tris, 50 mM), and NaCl (100 mM). The pH of the reaction was measured before and after the experiment. The assays were started by adding the enzyme. In the absence of the enzyme, the rate of absorbance change was negligible. The concentrations of DHFR, NADPH and DHF were 3 nM, 100 μM and 15 μM, respectively. One unit (U) is defined as the amount of enzyme required to convert 1 μmol of DHF to tetrahydrofolate (THF) in 1 min at 25°C. The values of the maximum steady-state rate (Vmax) and the Michaelis constant of DHFR for DHF (KmDHF) and NADPH (KmNADPH) were determined from the curvature evident in plots of disappearance of NADPH and DHF versus time (10 determinations). For KmDHF or KmNADPH determination, the initial concentration of saturating NADPH (100 μM) or DHF (200 μM) was considered as constant until the complete consumption of 10 μM DHF or 20 μM NADPH by the enzyme (3 nM), respectively. Data were fitted by nonlinear regression to the integrated form of the Michaelis equation,24 using Marquardt’s algorithm implemented in Sigma Plot 8.02 for Windows (Sigma Plot SPSS, Chicago).

**DHFR inhibition experiments and kinetic data analysis**

Initial velocity inhibition experiments were carried out on *C. albicans* DHFR with EGCG. The concentration of inhibitor that reduces enzyme velocity by half is defined as IC₅₀ and was determined by representations of the residual activity of the enzyme at different concentrations of EGCG in reaction mixtures containing 100 μM NADPH, 15 μM DHF and 3 nM enzyme. For Ki determination, one substrate (NADPH) was held constant at saturating concentration, while the other substrate (DHF) and the inhibitor (EGCG) were varied. To prevent the oxidation of EGCG the reaction mixture contained 1 mM N-acetylcysteine (Sigma). The extent of recovery of enzymatic activity following inhibition induced by preincubation with DHFR inhibitors was determined as follows. DHFR (0.15 μM) was preincubated for 30 min at 25°C in buffer containing EGCG. An aliquot of the incubation mixture was then diluted 50-fold into a reaction mixture containing buffer, NADPH (100 μM) and DHF (15 μM). The recovery of enzyme activity was followed by continuous monitoring at 340 nm.

**Broth dilution MIC determination**

MICs for all the strains were determined by the broth dilution method according to the M27-A broth microdilution reference procedure of the NCCLS20 at a final inoculum of 0.5 × 10⁷ to 2.5 × 10⁸ cfu/mL.
using RPMI 1640 medium with 2% glucose (Sigma-Aldrich). Final inoculum was verified by plating in duplicate 100 μL of a 100-fold saline dilution onto Sabouraud dextrose agar. After aerobic incubation at 35°C for 24 h, the lowest concentration of drug that produced a prominent decrease in turbidity (2 on a scale of 1–4) compared with that of the drug-free control was defined as its MIC.

Chequerboard synergy testing

Chequerboard tests were performed for all isolates by broth dilution in RPMI 1640 medium broth (Life Technologies, Inc., Barcelona, Spain) with 2% glucose combining eight doubling concentrations of EGCG with another eight dilutions of azole compounds (itraconazole and ketoconazole) or terbinafine. Inoculum was prepared by suspending yeast growth from Sabouraud agar plates in sterile saline to a final density equivalent to that of a 0.5 McFarland standard and diluted in RPMI 1640 medium broth to a final inoculum of 0.5 × 10⁵ to 2.5 × 10⁷ cfu/mL. Tubes were incubated aerobically overnight at 35°C. Fractional inhibitory combinations (FICs) were calculated as the MIC of antifungal and EGCG in combination divided by the MIC of the antifungal or EGCG alone, while the FIC index was obtained by adding the FIC values. FIC indices were defined as synergistic when values were ≤0.5 and antagonistic when values were >4. The results between synergy and antagonism were defined as additive or indifferent.

Reversion experiments with leucovorin or SAM

The 12 clinical isolates and the ATCC strain (final inoculum 0.5 × 10⁵ to 2.5 × 10⁷ cfu/mL) were grown on 96-well microplates containing ketoconazole or EGCG (at a concentration MIC/2 for each isolate) in cation-adjusted Mueller–Hinton broth with and without 0.5 mM leucovorin (Sigma) or different concentrations of S-adenosylmethionine (SAM) (Sigma). Control experiments contained no antifungal. The plates were sealed and incubated aerobically at 35°C for 24 h, after which time absorbance at 405 nm was read in a microplate spectrophotometer (SPECTRAmax, 340PC 384, Molecular Devices Corporation, CA).

Ergosterol quantification method

Total intracellular sterols were extracted as reported by Breivik and Owades27 with slight modifications. Briefly, a single C. albicans colony from an overnight Sabouraud dextrose agar plate culture was used to inoculate each of the flasks containing 10 mL of YPD broth, made with 20 g/L tryptone, 10 g/L yeast extract (both from Oxoid LTD, Basingstoke, Hampshire, UK) and 20 g/L dextrose (SA Probus, Barcelona), and enriched with different concentrations of antifungal compounds in the absence or presence of leucovorin (0.5 mM). The cultures were incubated for 16 h with shaking at 35°C. The stationary-phase cells were harvested by centrifugation at 2700 rpm for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellet was determined. Three millilitres of 25% alcoholic potassium hydroxide solution (25 g of KOH and 35 mL of sterile water, brought to 100 mL with 100% ethanol) was added to each pellet and vortex mixed for 1 min. Cell suspensions were transferred to sterile tubes and incubated in an 85°C water bath for 1 h. Following incubation, tubes were allowed to cool to room temperature. Sterols were then extracted by adding a mixture of 1 mL sterile water and 3 mL n-hexane followed by vigorous vortex mixing for 3 min. The hexane layer was transferred to a clean tube and stored at –20°C until use. For analysis, a 20 μL aliquot of sterol extract was diluted 5-fold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm with a Perkin-Elmer Lambda-35 spectrophotometer. The presence of ergosterol and the late sterol intermediate 24(28)-dehydroergosterol [24(28)DHE] in the extracted sample resulted in the characteristic four-peaked spectra.28 The ergosterol content was calculated as a percentage of the wet weight of the cell by the following equations:

\[
\% \text{ergosterol} + \% \text{24(28)DHE} = \left(\frac{A_{248} \times 520}{A_{251} \times 520}\right) \times \frac{\text{pellet weight}}{\text{pellet weight} + \text{pellet weight}} \times 100
\]

C. albicans was cultured overnight in RPMI 1640 medium with 2% glucose on 35 mm glass bottom microwell dishes. At zero time, cells were treated with 50 mg/L methotrexate-fluorescein (Molecular Probes, Invitrogen) and preparations were visualized each 60 min in a Leica TCS 4D confocal scanning laser fluorescence inverted microscope at 63-fold magnification.

Results

Antimycotic action of EGCG on C. albicans

In order to elucidate the mechanism of action of this antimycotic drug, we have determined the MICs of EGCG against 12 clinical isolates of C. albicans and an ATCC strain (ATCC 10231) at pH 7.4 (Table 1). The MICs for the strains tested ranged from 1 to 32 mg/L, which is within the range of previous reported data.13 The antifungal effect of EGCG has been found to depend on pH.13 EGCG was more active at slightly basic pHs with mean MIC varying from 1024 mg/L at pH 6.0 to 2.0 mg/L at pH 8.0.

Inhibition studies of C. albicans DHFR by EGCG

To determine if the antimycotic action of EGCG on C. albicans was due to the inhibition of DHFR, we purified this enzyme from isolate ARX1. The enzyme was then kinetically characterized and its in vitro inhibition by EGCG was studied. Calculation of the Michaelis constant for dihydrofolate (DHF) and NADPH were measured. Calculation of the Michaelis constant for dihydrofolate (DHF) and NADPH were measured.
inhibition of \( C.\) albicans DHFR by EGCG at pH 7.4. This compound affected the initial rate of DHFR reaction with its substrates, NADPH and DHF, with an IC\(_{50}\) of 5.0 \( \mu M\) (Figure 1a, inset). Double-reciprocal plots at saturating concentrations of NADPH and variable concentrations of DHF and EGCG showed a set of straight lines, which intercept on the ordinate axis (data not shown). Preincubation experiments of the enzyme in the presence of different concentrations of EGCG did not show any effect on enzyme activity. These results are characteristic of reversible and competitive inhibition with respect to DHF, with a calculated inhibition constant (\( K_i \)) of 0.7 \( \mu M\). Therefore, the data indicated that EGCG could act as an antifolate compound. Moreover, this inhibition was found to be highly modulated by pH, with EGCG being more active at slightly basic pH (Figure 1b).

Comparative activity of EGCG combined with other agents

Additional evidence of the antifolate activity of EGCG against \( C.\) albicans was obtained from ‘rescue’ experiments with folinic acid (leucovorin). Leucovorin is an active form of folic acid and is used as an antidote to drugs that decrease cellular levels of folic acid. Leucovorin is used in combination with trimethoprim to prevent bone marrow toxicity and with methotrexate in cancer chemotherapy. Antifolates block the de novo biosynthesis of thymine, purines and pyrimidines by inhibiting the synthesis of THF, an essential cofactor in these biosynthetic pathways. The survival rate of cells growing with leucovorin increases in the presence of antifolate compounds. Figure 2(a) shows the percentage of \( C.\) albicans growth after 18 h incubation with EGCG in the absence and presence of leucovorin. \( C.\) albicans grown in RPMI 1640 medium enriched with 0.5 mM leucovorin showed a high level of inhibition reversal in the presence of EGCG. Although the data presented above indicated that EGCG could act as an antifolate compound, the results obtained in our laboratory and those of others\(^{13}\) indicated that EGCG could also interact with ergosterol synthesis. Individual MICs

### Table 1. Susceptibility of \( C.\) albicans to tested antimycotics and chequerboard synergy test for EGCG with azoles at pH 7.4

<table>
<thead>
<tr>
<th>Strain/isolate</th>
<th>Susceptibility [MIC (mg/L)]</th>
<th>EGCG/ITZ</th>
<th>EGCG/KTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (mg/L)</td>
<td>FIC index (interpretation)(^a)</td>
<td>MIC (mg/L)</td>
</tr>
<tr>
<td>ATCC 10231</td>
<td>0.06/0.01</td>
<td>0.29 (S)</td>
<td>0.5/0.008</td>
</tr>
<tr>
<td>ARX1</td>
<td>0.03/0.007</td>
<td>0.50 (S)</td>
<td>0.06/0.015</td>
</tr>
<tr>
<td>ARX2</td>
<td>1/0.004</td>
<td>0.64 (A)</td>
<td>0.13/0.002</td>
</tr>
<tr>
<td>ARX3</td>
<td>1/0.03</td>
<td>0.31 (S)</td>
<td>0.13/0.01</td>
</tr>
<tr>
<td>ARX4</td>
<td>1/0.004</td>
<td>0.32 (S)</td>
<td>0.25/0.002</td>
</tr>
<tr>
<td>ARX5</td>
<td>0.25/0.06</td>
<td>0.31 (S)</td>
<td>0.03/0.25</td>
</tr>
<tr>
<td>ARX6</td>
<td>1/0.004</td>
<td>0.64 (A)</td>
<td>0.13/0.002</td>
</tr>
<tr>
<td>ARX7</td>
<td>0.5/0.01</td>
<td>0.25 (S)</td>
<td>0.25/0.007</td>
</tr>
<tr>
<td>ARX8</td>
<td>0.25/0.003</td>
<td>0.33 (S)</td>
<td>0.25/0.003</td>
</tr>
<tr>
<td>ARX9</td>
<td>0.25/0.062</td>
<td>0.18 (S)</td>
<td>0.25/0.06</td>
</tr>
<tr>
<td>ARX10</td>
<td>1/0.004</td>
<td>0.42 (S)</td>
<td>0.25/0.004</td>
</tr>
<tr>
<td>ARX11</td>
<td>0.25/0.008</td>
<td>0.32 (S)</td>
<td>0.13/0.006</td>
</tr>
<tr>
<td>ARX12</td>
<td>0.25/0.003</td>
<td>0.29 (S)</td>
<td>0.25/0.002</td>
</tr>
</tbody>
</table>

ITC, itraconazole; KTC, ketoconazole.

\(^a\)S, synergy; A, additive.
against *C. albicans* for two different azoles (itraconazole and ketoconazole) are shown in Table 1. Although both compounds share the same molecular target, sterol 14α-demethylase, in the ergosterol biosynthesis pathway; they present different chemical structures and activity profiles with different resistance patterns. MICs ranged from 0.01 to 16 mg/L and 0.01 to 4 mg/L for itraconazole and ketoconazole, respectively, and only one isolate (ARX7) was found to be resistant to bothazole drugs. Chequerboard titrations carried out in our laboratory revealed that the ATCC strain and all the isolates showed synergy between EGCG and ketoconazole, and only two of the 13 strains (ARX2 and ARX 6) showed an additive effect for treatment with the pair EGCG/itraconazole (Table 1). No FIC indices indicating antagonism were observed for any of the isolates. As expected, inhibition of *C. albicans* by anazole compound, such as ketoconazole, was not affected by the presence of leucovorin in the reaction medium (Figure 2a). To ascertain whether EGCG interfered with the synthesis of ergosterol, we showed that this compound acts synergistically with terbinafine, an inhibitor of another step in this metabolic pathway (squalene epoxidase) (Figure 2b).

**Figure 2.** (a) Effects of EGCG and ketoconazole (KTC) on *C. albicans* growth after 24 h of incubation in the presence and absence of 0.5 mM leucovorin. The data are expressed assuming 100% growth for the untreated control. Bars represent the average growth for the 13 isolates, and the error bars represent the standard deviations of the data. (b) The effect of the combination of terbinafine (TRB) with EGCG on the growth of *C. albicans* ARX1. The data are expressed assuming 100% growth for the untreated control. Bars represent the average growth for three individual experiments, and the error bars represent the standard deviations of the data.

Effects of EGCG in *C. albicans* ergosterol production

To demonstrate that EGCG could inhibit ergosterol synthesis, the content of this membrane component in *C. albicans* was examined after treatment with different concentrations of EGCG compared with untreated controls. The inhibition of ergosterol biosynthesis in *C. albicans* by EGCG is evident in Figure 3(a) and Table 2. A dose-dependent decrease in ergosterol production was observed when *C. albicans* strains were grown in the presence of EGCG (Table 2). EGCG at 2 mg/L inhibited ergosterol synthesis by about 50%. To check whether the inhibitory effect of EGCG on *C. albicans* ergosterol synthesis was related to its antifolate activity, we carried out a similar experiment, but in the presence of leucovorin. This compound was able to completely reverse EGCG-induced ergosterol inhibition (Figure 3b, Table 2). In order to show that the effects of EGCG and leucovorin on ergosterol biosynthesis are specific, control experiments with ketoconazole alone or in combination with leucovorin were carried out (Table 2). As expected, ketoconazole inhibits ergosterol biosynthesis in a dose-dependent manner, but this inhibition was not affected by the presence of leucovorin in the growth medium. Additional control experiments included an examination of the effects of 5-fluorocytosine on ergosterol biosynthesis. This antifungal compound inhibits both DNA and RNA synthesis via intracytoplasmic conversion to 5-fluorouracil. The latter is converted into two active nucleotides: 5-fluorouridine triphosphate, which inhibits RNA processing, and 5-fluorodeoxyuridine monophosphate, which inhibits thymidylate synthetase, and hence the formation of the deoxythymidine triphosphate needed for DNA synthesis. As predicted from its action mechanism, this compound had no effect on ergosterol production following a similar exposure concentration relative to its MIC (MIC = 0.03 mg/L) and duration (16 h) (Table 2).

Comparison of the antifungal action of EGCG with other antifolate compounds

In contrast to the situation with antibacterial agents, few antimetabolites are available for use against fungi. As mentioned in the Introduction, trimethoprim and methotrexate, two of the most commonly used antifolates in antimicrobial and anticancer therapies, show no antifungal activity (Table 3). EGCG, like trimethoprim and methotrexate, is a reversible and competitive inhibitor of *C. albicans* DHFR, but its inhibition constant is intermediate between these two antifolate compounds; EGCG ($K_i = 0.7 \mu M$) is a more effective inhibitor of *C. albicans* DHFR than trimethoprim ($K_i = 4.3 \mu M$) but less effective than methotrexate ($K_i = 150 \mu M$) at pH 7.4 (Table 3). To understand why a potent inhibitor of *C. albicans* DHFR such as methotrexate did not show much antifungal activity we used confocal microscopy to study the transport of this drug into the *C. albicans* cells. As can be seen in Figure 4, the fluorescent probe methotrexate-fluorescein did not cross the fungal membrane even after 6 h of treatment. This impermeability of *C. albicans* to methotrexate could explain the low activity of this drug.20,21 Another antifolate compound is the antimalarial agent pyrimethamine, which has about 10-fold greater affinity for the *C. albicans* enzyme than trimethoprim19 and an IC50 value similar to EGCG (Table 3). To check the antifungal activity of this compound we determined the MIC against the 13 strains used in this study (mean MIC = 32 mg/L). Like EGCG, pyrimethamine showed synergy with ketoconazole (data not shown; FIC = 0.3)
and interfered with ergosterol biosynthesis in similar concentrations relative to its MIC (Table 2). Moreover, the effects of pyrimethamine were also strongly reversed when leucovorin was included in the growth medium (Table 2). These data clearly show the connection between the folic acid cycle and the ergosterol biosynthesis pathway.

**Connection between the folic acid cycle and the ergosterol biosynthesis pathway in C. albicans**

Taken together the data indicated that EGCG, by disturbing folate metabolism in C. albicans cells, is able to inhibit ergosterol biosynthesis.
biosynthesis. What, then, is the connection between the folic acid cycle and the ergosterol biosynthesis pathway in this organism? By examining the pathway for ergosterol synthesis (Figure 5) a candidate for this link could be sterol C-24 methyltransferase (24-SMT), an enzyme dependent upon SAM. The efficacy of antifolates in treating cancer or bacterial infections is widely attributed to a decrease in the production of nucleotides. In addition to effects on nucleotide biosynthesis, antifolate treatment has been linked to a decrease in cellular methylation (Figure 5). The enzyme methylene-THF reductase (MTHFR) catalysed the reduction of 5,10-methylene-THF to 5-methyl-THF. This molecule is a substrate for the conversion of homocysteine to methionine, which, in turn, is a precursor of SAM. Methylation reactions use SAM as a methyl group donor to produce a methylated product and S-adenosylhomocysteine (SAH). SAH is broken down by SAH hydrolase into adenosine and homocysteine. In folate-deficient cells, including cells treated with antifolates, a depletion of 5-methyl-THF blocks the methylation of homocysteine. The resulting accumulation of homocysteine drives SAH hydrolase to catalyse the energetically favourable reverse reaction and synthesize SAH, a potent product inhibitor of cellular methyltransferases. Such a decrease in methylation has been overlooked as a mechanism for the antiproliferative effects of antifolates. We hypothesized that the depletion of SAM and/or the inhibition of 24-SMT by the disruption of folate metabolism in C. albicans cells treated with EGCG could be responsible for the synergy between EGCG and azoles (Table 1), as well as the inhibition of ergosterol production (Figure 3a, Table 2). In order to prove this hypothesis we carried out reversion experiments with different concentrations of SAM in C. albicans cultures treated with an inhibitory concentration of EGCG. Although SAM did not affect C. albicans growth at any concentration, Figure 6(a) shows that the antifungal action of EGCG can be significantly reversed (close to 80%) by restoring cellular pools of SAM in the cells. This reversion was maximized at SAM concentrations close to 125 μM. To discriminate whether EGCG produced its action by disturbing folic acid metabolism or by direct inhibition of 24-SMT as observed for protoberberines, a series of experiments were carried out to determine the effects of EGCG on the incorporation of L-[methyl-14C]methionine into C. albicans ergosterol. As shown in Figure 6(b), incorporation of L-[methyl-14C]methionine at C-24 of ergosterol was not affected by the presence of EGCG. Based on the results of both experiments described in Figure 6, it can be concluded that EGCG is

**Figure 4.** Confocal microscopy of C. albicans with 50 mg/L methotrexate-fluorescein after 0 (a) and 6 h (b) treatments. A colour version of this figure is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**Figure 5.** Schematic representation of the molecular connection between the folic acid cycle and the ergosterol biosynthetic pathway in C. albicans. The sites of action for azole drugs and EGCG are proposed. TS, thymidylate synthase; MS, methionine synthase.
not an inhibitor of 24-SMT, and therefore, inhibition of ergosterol synthesis in *C. albicans* (Figure 3a; Table 2) appears to be a consequence of the decrease in SAM levels, which result in a blockage of the 24-SMT step due to unavailability of substrate.

**Discussion**

Recently, it has been observed that the gallated tea polyphenol, EGCG, possesses antymycotic activity on *C. albicans*. To elucidate its molecular mechanism of action we have shown that EGCG is an antifolate compound. The antifolate character of EGCG is evident from its similarity to the action mechanism of the non-classical antifolate, pyrimethamine. Both inhibit *C. albicans* DHFR in a fast reversible competitive process and are synergistic with respect to azole compounds, while fungal growth inhibition in the presence of these drugs is reversed by growing *C. albicans* in a medium enriched with leucovorin. Antifolates inhibit DHFR, preventing the regeneration of THF, and bringing the folate cycle to a halt. The resulting lack of dTTP causes a nucleotide imbalance, which leads to the misincorporation of nucleotides into the DNA and eventually to the death of the cell (Figure 5). Moreover, antifolates have also been implicated in the decrease of cellular methylation. Although we present data that EGCG is a potent inhibitor of *C. albicans* DHFR in vitro, other enzymes of the folate cycle could be targets of EGCG in vivo, and so, other enzymes of the folate cycle should looked at as possible targets of EGCG.

As observed for bovine liver DHFR, EGCG is a better inhibitor of *C. albicans* DHFR at slightly basic pH. The molecular basis for this pH-dependent inhibition of DHFR by EGCG has been discussed recently. The pH controls both the ionization of critical catalytic residues of the enzyme and the protonation state of EGCG (pK 7.8) and, therefore, the effective concentrations of the protonated and unprotonated forms. Each form will exhibit a different affinity and interaction with different molecular target(s) responsible for the cellular effects of EGCG. Thus, for DHFR inhibition, the deprotonated form has been proposed as the more effective inhibitor. Arg-70 in human DHFR (a highly conserved residue also found in the *C. albicans* enzyme) has been shown by X-ray crystallography to interact with the α-carboxylate of the terminal L-glutamate moiety of folic acid or MTX. Mutation of this residue was found to result in insensitivity to MTX. Structural modelling of EGCG into human DHFR indicates that the compound is oriented in a similar way to MTX or folic acid, with the ionizable ester-bonded gallate moiety close to Arg-70. The pH-dependent ionization of this gallate could favour the formation of a hydrogen bond or electrostatic interaction with Arg-70. The implication of Arg-70 on inhibitor binding in *C. albicans* DHFR has also been reported. Additionally, it appears that the ionization state of an essential residue (probably Glu-30) is critical for the catechin-dependent inhibition of the bovine enzyme. In fact, when this residue is protonated, EGCG loses its efficiency as an inhibitor of DHFR. Importantly, Glu-32 is also a critical residue in *C. albicans* DHFR. More difficult to confirm is whether this pH-dependence of the inhibition of *C. albicans* DHFR in vitro is related with the effect that the pH has been found to have on its antifungal activity. These dependencies are important, especially for an organism such as *C. albicans* because its ability to respond to ambient pH appears to play a critical role in growth and virulence. Many of the genes required for virulence in fungal pathogens are regulated in response to environmental signals indigenous to the host niche. It is known that external pH and temperature influence, at least in *vivo*, the yeast-to-mycelium transition of *C. albicans*. It is well known that *C. albicans* has a fast response to environmental changes. It was determined that when *C. albicans* was exposed to conditions that produced yeast-to-mycelium transitions (including increase in external pH) the cytoplasmic pH of this organism increased from 6.5 to over 8.0 within 5 min. Thus, small changes in the intracellular pH could be important for catechin’s action; however, other factors not affecting DHFR and regulated by the extracellular pH, such as growth rate, morphology or drug permeability, could also be involved in the pH response of *C. albicans* to EGCG.

From a biochemical point of view, perhaps the most interesting finding in this study is the identification and understanding of the molecular mechanism for the synergy between EGCG andazole antifungals. EGCG can indirectly disrupt the ergoster synthesis pathway through disruption of the folate cycle. EGCG caused the inhibition of ergoster biosynthesis due to the reduction of cellular pools of SAM, which produces a blockage of this pathway through the inhibition of 24-SMT. The physiological importance of the blockage of this step of the
EGCG inhibits C. albicans DHFR

ergosterol biosynthesis pathway is under discussion. Although it has been clearly shown that protoberberines, the specific inhibitors of 24-SMT, cause a significant inhibition of growth of Candida species,29 published genetic data indicated that deletion of the ERG6 gene (encoding for 24-SMT) in C. albicans was no more sensitive to azole drugs than the wild-type.46 The data presented here clearly show the importance of the SAM pools in the cells for an efficient synthesis of ergosterol in C. albicans. Doubtlessly, the viability of mutants with no 24-SMT is due to the apparition of new survival mechanisms in the presence of appropriate cellular pools of SAM. Thus, the response of these mutants to different antifungal compounds could differ from the response of the wild-type yeast to the drugs.

The target for azoles is sterol 14α-demethylelase, which is part of the ergosterol biosynthetic pathway (Figure 5). The resulting ergosterol depletion renders fungal cells vulnerable to further membrane damage. The development ofazole resistance in fungi may occur through increased levels of the cellular target, upregulation of genes controlling drug efflux, alteration in sterol synthesis and decreased affinity ofazole for the cellular target.30 Some of the problems associated with these resistance mechanisms could be solved by introducing a second target enzyme in the ergosterol biosynthesis pathway, e.g. 24-SMT, by using EGCG. In fact, we observed a good level of EGCG activity on the most sterol-resistant isolate (ARX7) used in this work. The clinical relevance of these in vitro results will need to be confirmed by investigation of their therapeutic efficacy. Further studies with a higher number of azole-resistant strains should help elucidate whether EGCG maintains a good level of activity in all cases. If this is the case, this drug, alone or in combination with azoles, could represent an alternative for the treatment of C. albicans infections.

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Transparency declarations

Nothing to declare.

Supplementary data

A colour version of Figure 4 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


