INTRODUCTION

Candida albicans is a commensal organism found in the human gastrointestinal tract and vaginal mucosa, and is one of the major human pathogenic fungi. It is recovered frequently from immunocompromised hosts such as AIDS, cancer or organ transplant patients. Previous studies on the susceptibility of yeast isolated from cancer patients being treated with antineoplastic drugs suggested that these agents can reduce the susceptibility to polyenic drugs (Dick et al., 1980; Ahearn & McGlohn, 1984; Brajtburg et al., 1988; Sarachek & Henderson, 1991; Abrahamsen et al., 1992; Conly et al., 1992; Vanden Bossche et al., 1994; Salonen et al., 2000; Davies et al., 2002).

Among the biochemical mechanisms that must be studied to explain the low susceptibility to amphotericin B is the increased catalase activity. Catalase is an important enzymatic mechanism against oxidative damage (Sokol-Anderson et al., 1988). The target structure for amphotericin B is the plasma membrane ergosterol, where the polyenic drug forms a channel. Through this channel the fungal cell leaks potassium ions, resulting in a disruption of the proton gradient. It is hypothesized that rings of 8–10 polyeone molecules form aqueous pores within the membrane bilayer structure, and amphotericin B causes oxidative damage to plasma membranes (Sokol-Anderson et al., 1986; Kerridge & Nicholas, 1986; Nakagawa et al., 2003).

The treatment of candidiasis in cancer and/or transplant patients is difficult, and frequently is a failure. The emergence of Candida resistance in this context requires new evaluations, like the study of determinant factors in the emergence of resistance, among them antineoplastic therapy. Studies aiming to evaluate the catalase activity with regard to antineoplastic agents are either scarce or not complete. In view of this, we have analysed C. albicans catalase activity when the yeast is exposed to antineoplastic drugs at the same therapeutic concentrations as are used in human plasma.

The catalase activity of C. albicans exposed to the drugs was compared to the same group not exposed to drugs. To demonstrate the kinetics of antineoplastic drugs capable of changing the catalase activity of C. albicans, $K_m$ and $V_{max}$ were determined by Lineweaver–Burk analyses.

METHODS

Strains. C. albicans strains used in this study are listed in Table 1. Cultures of C. albicans were isolated from patients treated at University Hospital of Santa Maria, Santa Maria, Brazil who had no previous contact with the antineoplastic drugs. HIV-positive patients were chosen due to the ease of isolating opportunist fungi without invasive methods. The identification of the species was based on their phenotypic characteristics. Only numbers were used to identify the yeast strains, shown in Tables 1 and 2. C. albicans from the American Type Culture Collection, ATCC 44373, was used as the control strain. The protocol used was approved by the Bioethics
Table 1. Yeast strains

Data are the means ± SD of triplicate experiments.

<table>
<thead>
<tr>
<th>Yeast*</th>
<th>Drug-free yeast†</th>
<th>Yeast exposed to dacarbazine‡§</th>
<th>Yeast exposed to cyclophosphamide¶§</th>
<th>Yeast exposed to cytarabine¶§</th>
<th>Yeast exposed to methotrexate#</th>
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<tr>
<td>10</td>
<td>3·6 ± 1·1</td>
<td>5·9 ± 0·1</td>
<td>5·5 ± 1·7</td>
<td>6·1 ± 1·6</td>
<td>14·9 ± 3·6</td>
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<tr>
<td>172</td>
<td>5·2 ± 1·9</td>
<td>9·5 ± 3·1</td>
<td>9·6 ± 2·8</td>
<td>6·7 ± 0·8</td>
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<td>7·7 ± 1·2</td>
<td>13·1 ± 4·1</td>
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<tr>
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<td>4·0 ± 0·4</td>
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<td>59</td>
<td>4·2 ± 1·6</td>
<td>13·8 ± 2·3</td>
<td>15·0 ± 3·8</td>
<td>5·5 ± 2·2</td>
<td>10·8 ± 0·7</td>
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<td>7·4 ± 0·6</td>
<td>10·0 ± 0·6</td>
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<td>9·6 ± 2·9</td>
<td>8·5 ± 0·4</td>
<td>4·5 ± 1·1</td>
<td>22·8 ± 2·2</td>
</tr>
<tr>
<td>ATCC 44373</td>
<td>6·7 ± 2·2</td>
<td>5·4 ± 2·5</td>
<td>4·0 ± 1·6</td>
<td>3·0 ± 0·6</td>
<td>8·1 ± 1·1</td>
</tr>
</tbody>
</table>

*C. albicans* isolated from HIV-positive patients assisted in the University Hospital of Santa Maria.
†C. albicans not exposed to antineoplastic drug.
‡C. albicans exposed to dacarbazine (8 µg ml⁻¹).
§Statistical analysis did not demonstrate significant differences between C. albicans exposed and not exposed to the drugs (P > 0·05).
¶C. albicans exposed to cyclophosphamide (50 µg ml⁻¹).
§C. albicans exposed to cytarabine (1 µg ml⁻¹).
#C. albicans exposed to methotrexate (2 µg ml⁻¹). Statistical analysis showed very significant differences between C. albicans exposed and not exposed (P < 0·01).

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Table 2. Effect of methotrexate on C. albicans catalase activity

<table>
<thead>
<tr>
<th>Yeast*</th>
<th>Methotrexate†</th>
<th>KₐθK (mM)</th>
<th>VₐθK [AE min⁻¹ (mg protein)⁻¹]</th>
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<tr>
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<tr>
<td>124</td>
<td>0 µM</td>
<td>1·96</td>
<td>3·08</td>
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<td>2 µM</td>
<td>2·15</td>
<td>4·77</td>
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<td>8 µM</td>
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<td>4 µM</td>
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<tr>
<td></td>
<td>8 µM</td>
<td>2·55</td>
<td>4·62</td>
</tr>
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</table>

*C. albicans* that presented the highest differences between yeasts exposed and not exposed to methotrexate.
†Antineoplastic drug that produced significant differences in catalase activity in C. albicans.
‡Substrate concentration.
§Maximum velocity.

Antineoplastic exposition. The selection of the drugs was based on the most frequently used antineoplastic drugs for cancer treatment at the University Hospital of Santa Maria. The concentrations of cyclophosphamide (50 µg ml⁻¹) [genoxal – 2H-1,3,2-oxazaphosphorin-2-amine, N,N-bis(2-chloroethyl)-tetrahydro-2-oxide monohydrate], cytarabine (1 µg ml⁻¹) [ara-cytin – 4-amino-1-b-D-arabinofuranosyl-2(1H)-pyrimidinone], methotrexate (2 µg ml⁻¹ = 4 µM) [litrectase – N-[4-[[2,4-diamino-6-pteridiny]methyl]methylamino] benzoyl]-1-glutamic acid] and dacarbazine (8 µg ml⁻¹) [DTI – 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide] used in the tests were mimicking the therapeutic plasma concentrations of these drugs, which were obtained from other studies (Egorin et al., 1989; Lillemark & Peterson, 1991; Morikawa et al., 1997; Batey et al., 2002).

The strains were grown for 24 h in glucose Sabouraud agar and then were cultivated in 6 ml YNB (Yeast Nitrogen Base without amino acids; Difco), supplemented with the appropriate antineoplastic drug, for 48 h at 35°C. The tests were performed in triplicate.

Preparation of cell-free extracts. A crude extract was prepared by glass-bead lysis. Cells cultured in YNB supplemented with drug were washed three times with 0·85% saline and then resuspended in lysis buffer (50 mmol l⁻¹ potassium phosphate pH 7·0) containing 0·5 g 500 µm diameter glass beads. The mixture was homogenized in a Teflon-glass homogenizer by 4–6 cycles of 20 s alternating with lysis buffer (50 mmol l⁻¹ potassium phosphate pH 7·0) containing 0·5 g 500 µm diameter glass beads. The mixture was centrifuged for 20 min in a refrigerated centrifuge to remove cell debris and glass beads. The supernatant was used for enzyme assays.

Catalase activity. Catalase activity was determined in cell-free extracts by the method of Aebi (1984). The readings were performed in triplicate.

Protein analysis. Protein was measured by the Bradford method using Coomassie blue, and serum albumin as a standard (Bradford, 1976).

Kinetic analysis. The kinetics of the interaction between the antineoplastic drug and catalase were determined using a
Catalase activity in Candida

Lineweaver–Burk double reciprocal plot (Lineweaver & Burk, 1934), by plotting $1/v$ against $1/S$ analysed over a range of hydrogen peroxide concentrations (1–50 mmol l$^{-1}$) in the absence and in the presence of methotrexate (2–8 μM). $K_m$ values were obtained by two different estimates, $1/v$ vs $1/S$ and $1/v$ vs $v/S$.

Statistics. In order to compare the means obtained for catalase activity in the groups exposed and not exposed to antineoplastic drug, the $t$-test was used, using the GraphPad InStat statistical program.

RESULTS AND DISCUSSION

The emergence of fluconazole and amphotericin B resistant strains of C. albicans in cancer patients is a troubling new development (Nolte et al., 1997). Though over the last decade fluconazole-resistant Candida sp. infections in cancer patients were more studied than amphotericin B resistant infections, currently, the increasing incidence of development of amphotericin B resistance in patients with Candida sp. infection, and receiving antineoplastic and antifungal therapy, is a reality.

In this context, the increased fungal catalase activity has been suggested as a mechanism that not only reduces the amphotericin B activity, but also protects against oxidative damage, favouring the survival of fungal cells. This may be related to the resistance mechanism for amphotericin B (Sokol-Anderson et al., 1988). However, since 1990, very few complete studies have demonstrated the influence of antineoplastic drugs on catalase activity, a fact that impelled this study, especially considering the failure of systemic mycosis in cancer patients.

To verify the antineoplastic drug influence on catalase activity, non-exposed C. albicans was used as a control in this study. Table 1 shows the levels of catalase in C. albicans not exposed to drugs and exposed to dacarbazine [8 μg ml$^{-1}$], cyclophosphamide [50 μg ml$^{-1}$], cytarabine [1 μg ml$^{-1}$] and methotrexate [2 μg ml$^{-1}$ = 4 μM]. Statistical analysis demonstrated no significant differences ($P > 0.05$) between the groups of C. albicans exposed and not exposed to dacarbazine, cyclophosphamide and cytarabine. The mean catalase activity in yeast exposed to dacarbazine $[8.4 \pm 3.14 \, \Delta E \, \min^{-1} \, (mg \, protein)^{-1}]$ or cyclophosphamide $[9.137 \pm 3.6 \, \Delta E \, \min^{-1} \, (mg \, protein)^{-1}]$ was only slightly higher than in the non-exposed yeast $[6.412 \pm 3.73 \, \Delta E \, \min^{-1} \, (mg \, protein)^{-1}]$. The mean catalase activity in yeast exposed to cytarabine $[5.95 \pm 2.2 \, \Delta E \, \min^{-1} \, (mg \, protein)^{-1}]$ was slightly lower than that in the drug-free yeast.

Although no statistical differences were found in the groups exposed and not exposed to dacarbazine, cyclophosphamide and cytarabine, we observed that when we took C. albicans ATCC 44373 catalase activity as a standard, the yeasts with catalase activity lower than C. albicans ATCC 44373 increased their levels of catalase, and the yeasts with levels of catalase activity higher than C. albicans ATCC 44373 decreased their levels after exposure. Extensive investigations are necessary to demonstrate the real influence of dacarbazine, cyclophosphamide and cytarabine on C. albicans catalase activity and resistance to antifungal therapy.

The effect of methotrexate on catalase activity in C. albicans, shown in Table 1, was a significant difference ($P < 0.01$) in

![Fig. 1. Kinetic analysis of the catalase activation by methotrexate in yeast 124 (a), 170 (b) and 172 (c). The graphs show double reciprocal plots of the catalase experiments in the absence and in the presence of methotrexate concentrations ranging from 2 to 8 mM (○, 0 μM; ◆, 2 μM; □, 4 μM; ■, 8 μM). Catalase activity ($v$) was measured at various concentrations of substrate ($S$) (1.5–30 mM) in 2 ml assay solutions with 100 mM phosphate buffer (pH 7–0) using 200 μl lysed fungal cells. Experiments were repeated three times.](http://jmm.sgmjournals.org)
activity when compared to the drug-free yeast. The activity found in yeasts exposed to the drug [16-75 ± 7-96 ΔE min⁻¹ (mg protein)⁻¹] was higher than that in the drug-free yeasts [6-412 ± 3-73 ΔE min⁻¹ (mg protein)⁻¹]. In view of these catalase activity alterations, a kinetic study was performed for the yeasts that presented more alterations when exposed to this antineoplastic drug. The effects of methotrexate on $K_m$ and $V_{max}$ shown in Table 2, were determined by Lineweaver–Burk plots (Fig. 1). The kinetic study showed $K_m$ and $V_{max}$ increased when yeast 124 (Fig. 1a), 170 (Fig. 1b) and 172 (Fig. 1c) were exposed to increasing levels of methotrexate. For these yeasts the $V_{max}$ was more than double compared to that of yeasts exposed to 8 μM methotrexate (twice plasma concentration). Increasing $K_m$ values were found for the three yeasts when exposed to increasing methotrexate concentrations compared to the same group not exposed. The Lineweaver–Burk kinetic study with the three yeasts showed an uncompetitive pattern. Probably, the concentration of the reactants produced a large rate of interaction between the enzyme and the substrate causing an elevation of the catalase $V_{max}$ and consequently of the formation of the product. This fact may contribute to the appearance of amphotericin B resistance in cancer patients undergoing antineoplastic drug treatment.

In accordance with our results, Brajtburg et al. (1988) found an increased level of catalase was induced by 2-chloroethyl-1-nitrosoureas, an important drug in the therapy of cancer. Ahearn & McGlohn (1984) suggested that cytotoxic drugs might be responsible for mutations capable of producing resistance to polyenic drugs and consequent treatment failure in cancer patients with candidiasis.

Other studies involving Candida spp. and the antimetabolic agent methotrexate have been carried out by Ghannoum (1986), who observed that this antineoplastic agent elicited pseudomyceial formation in Candida glabrata. It was surprising because the inability of C. glabrata to form pseudomyceila is used as an important criterion in its identification. The formation of pseudohypha suggested a blockage of the cellular division mechanism in this yeast. However, a real interference of methotrexate in the antioxidant activity of C. albicans is being shown here for what is believed to be the first time.

REFERENCES


