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Antifolates as antimycotics? Connection between the folic acid cycle and the ergosterol biosynthesis pathway in *Candida albicans*

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Abstract

The increased incidence of invasive mycoses and the emerging problem of antifungal drug resistance have encouraged the search for new antifungal agents or effective combinations of existing drugs. Infections due to *Candida albicans* are usually treated with azole antifungals such as fluconazole, ketoconazole or itraconazole. Whilst azoles may 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. Here we demonstrate that the non-classical antifolate pyrimethamine shows synergy with azole antifungal compounds and interferes with the ergosterol biosynthesis pathway in *C. albicans*. By disturbing folate metabolism in this fungus, pyrimethamine can inhibit ergosterol production. The molecular connection between the folic acid cycle and the ergosterol biosynthesis pathway is discussed and we show that the filamentous form of this fungus is more susceptible to methotrexate than the yeast form because the drug is more effectively transported through the membrane of the filamentous form. When used to treat the hyphal form, methotrexate showed synergy with other antifungals such as azoles and terbinafine. This finding could have important clinical applications, as a combination of azoles with antifolates and/or inhibitors of folic acid synthesis could represent an attractive alternative for the treatment of *C. albicans* infections.

Keywords: Antifolates; Ergosterol; Folic acid; Dihydrofolate reductase; *Candida albicans*; Candidosis

1. Introduction

Candida albicans is a dimorphic fungus that exists most of the time as single, oval yeast cells, which reproduce by budding. Most yeasts do not produce mycelia (a mass of branching, thread-like, hyphal filaments), but under physiological conditions (body temperature, pH and the presence of serum) *Candida* can develop into a hyphal form. In the yeast state, *C. albicans* is a non-invasive, sugar-fermenting organism, whilst in the fungal state it is invasive and can produce rhizoids (very long root-like structures). Rhizoids can penetrate the mucosa or intestinal wall, leaving microscopic holes and allowing toxins, undigested food particles, bacteria and yeast to enter the bloodstream. Thus, *C. albicans* is a common cause of vaginitis and, in immunocompromised individuals, of oropharyngeal and systemic infections. Infections due to *C. albicans* are usually treated with azole antifungals such as fluconazole, ketoconazole and itraconazole [1] that inhibit the enzyme sterol 14α-demethylase in the sterol biosynthetic pathway [2,3]. This pathway is conserved in eukaryotes, leading to production of cholesterol in mammals and ergosterol in fungi. In *C. albicans*, sterols have been shown to be important for membrane fluidity, membrane permeability, cell morphology, enzyme activity and cell cycle progression [4–6]. However, whilst azoles have little or no toxicity, they generally show rather poor fungicidal activity. Even in the absence of resistance, treatment failures or recurrent infections are

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not uncommon, especially in immunocompromised individuals [7,8]. These clinical limitations associated with azole use have led to the search for novel antifungal drugs.

Dihydrofolate reductase (EC 1.5.1.3) (DHFR) is a key enzyme in the biosynthesis of purines, pyrimidines and several amino acids [9] and is the target of a number of drugs, including the antimicrobial agent trimethoprim (TMP) and the anticancer drug methotrexate (MTX). Inhibitors of folate metabolism, also called antifolates, are not effective in the treatment of *C. albicans* infections. In fact, *C. albicans* is a eukaryotic organism and expresses a TMP-insensitive DHFR that is closely similar to the mammalian enzyme. On the other hand, MTX, which is a potent inhibitor of *C. albicans* DHFR [10], shows no detectable anticandidal activity [11]. Recently, we have shown that a tea polyphenol, epigallocatechin-3-gallate, inhibited ergosterol biosynthesis by disturbing folic acid metabolism in *C. albicans* [12]. This finding encouraged us to study the factors that govern the action of antifolates on *C. albicans* and to look for molecular connections between folic acid metabolism and other pathways involved in *C. albicans* survival. The findings encouraged us to postulate the possibility of using these compounds as antimycotic agents.

2. Materials and methods

2.1. Microorganisms and growth conditions

Candida albicans ATCC 10231 and 19 clinical isolates were used in this study. The clinical isolates were collected over a 6-month period at the Hospital Universitario Virgen de la Arrixaca (Murcia, Spain). All strains were maintained routinely on Sabouraud dextrose agar (SDA) (Oxoid Ltd., Basingstoke, UK). The yeast form was obtained after growth in SDA at 25 °C for 24 h and the filamentous form after growth in RPMI-1640 medium with 2% glucose (RPMI-G; Sigma Chemical Co., Madrid, Spain) at 37 °C for 16 h to achieve 10^9 cells/mL.

2.2. Antifolates and antifungals

TMP, MTX and pyrimethamine (PYR) were obtained from Sigma. Ketoconazole (KTZ) was also obtained from Sigma. Stock solutions (5 g/L) were prepared in dimethyl sulfoxide (DMSO) and stored for a maximum of 2 months at −20 °C until use. Terbinafine (TRB) (Novartis, Barcelona, Spain) was dissolved in DMSO (12.8 g/L), diluted 2× in DMSO and then diluted 50× in complete medium. Amphotericin B (AMPH), 5-fluorocytosine (5-FC) and sulfadiazine (SPZ) were purchased from Sigma.

2.3. Minimum inhibitory concentration (MIC) determination by broth dilution

MICs for all the strains were determined by the broth dilution method according to the M27-A2 broth microdilution reference procedure of the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) [13] at a final inoculum of 0.5×10^5 to 2.5×10^5 colony-forming units (CFU)/mL using RPMI-G. The final inoculum was verified by plating (in duplicate) 100 μL of a 100-fold saline dilution onto SDA. After aerobic incubation at 35 °C for 16 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 the MIC for azole compounds or 5-FC. Following CLSI recommendations for antifungal tests [13], the MICs of the other compounds were determined as the lowest concentration of drug that provided visual growth inhibition.

2.4. Checkerboard synergy testing

Checkerboard tests were performed for all the isolates in their yeast or filamentous form (final inoculum of 0.5×10^5 to 2.5×10^5 CFU/mL) by dilution in RPMI-G combining eight doubling concentrations of antifolates (MTX or PYR) or SPZ with another eight dilutions of antifungals (KTZ, TRB, AMPH or 5-FC) in 96-well microplates. The plates were sealed and incubated aerobically for 16 h at 35 °C.

2.5. Reversion experiments with leucovorin or S-adenosylmethionine (SAM)

The 19 clinical isolates and strain ATCC 10231 (final inoculum 0.5×10^5 to 2.5×10^5 CFU/mL) were grown in 96-well microplates containing different concentrations of PYR in RPMI-G with and without leucovorin (0.5 mM) or SAM (0.25 mM). Control experiments contained no antifungal agent. The plates were sealed and incubated aerobically at 35 °C for 16 h, after which absorbance at 405 nm was read using a microplate spectrophotometer (SpectraMax, 340PC[38]; Molecular Devices Corporation, Sunnyvale, CA).

2.6. Ergosterol quantification method

A single *C. albicans* colony from an overnight SDA 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, UK) and 20 g/L dextrose (Probus S.A., Barcelona, Spain). Different concentrations of antifungal compounds were added in the absence or presence of leucovorin (0.5 mM). The cultures were incubated for 16 h with shaking at 35 °C. 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. Total intracellular sterols were extracted and quantified as described elsewhere [12].

2.7. Confocal microscopy

The yeast form of *C. albicans* was cultured overnight in RPMI-G over 35 mm glass-bottomed microwell dishes.