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Ketoconazole Binds to the Intracellular Corticosteroid-Binding Protein in Candida albicans+

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Ketoconazole is a broad-spectrum, orally-active antifungal agent that has been shown to inhibit sterol synthesis in susceptible fungi. We have previously demonstrated the presence of an intracellular protein in several Candida species that binds mammalian corticosteroids with high affinity. In this paper we report that ketoconazole competitively displaces [3H]corticosterone from the Candida corticosteroid-binding protein at concentrations readily achieved in therapeutic settings. Ketoconazole was at least 50-100 times more potent than structurally related imidazole compounds. Additional data suggest, however, that the binding of ketoconazole and related drugs to this Candida protein is not critical for the in vitro antifungal activity of these drugs.

Ketoconazole is an important new imidazole antifungal agent. The drug can be administered orally, it produces minimal side effects, and it possesses a broad spectrum of activity against pathogenic fungi, including Candida species (1). An understanding of the mechanism of action of ketoconazole and the imidazole antifungals remains imcomplete; however, available evidence supports the hypothesis that a primary action of these agents is the impairment of sterol synthesis in susceptible fungi, leading to defective membrane synthesis (2). Additional actions, including direct effects on membrane-bound enzymes, have also been postulated (2-4).

We have previously demonstrated the existence of an intracellular protein in several Candida species that binds mammalian corticosteroids (5-7). This Candida corticosteroid-binding protein (CBP)[1] binds corticosterone and progesterone with high affinity and specificity. We have also provided evidence that C. albicans possesses an endogenous ligand which appears to bind to both Candida CBP and

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[1]The abbreviation CBP is used for corticosterone binding protein and MIC for minimal inhibitory concentration.

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mammalian glucocorticoid receptors (5). These findings have led us to hypothesize that C. albicans has a hormone-receptor system that may modulate the physiology and/or the pathogenicity of the fungus. In recent studies of ketoconazole interactions with mammalian tissues, we reported that ketoconazole binds to glucocorticoid receptors and thereby exhibits glucocorticoid antagonist activity in hepatoma tissue culture cells (8). Since corticosteroids bind to Candida CBP (5-7), we decided to examine the possibility that ketoconazole might also bind to this Candida protein. We now report that ketoconazole and structurally-related imidazole compounds displace [^{3}H]corticosterone from Candida CBP.

Materials and Method

[^{3}H])Corticosterone (57 Ci/mmol) was purchased from Amersham (Arlington Heights, IL.). Radioinert corticosterone was obtained from Steraloids (Wilton, N.H.). Ketoconazole (free base) was a gift from Janssen Pharmaceutica (Beerse, Belgium). Clotrimazole was a gift from Schering (Bloomfield, NJ), and imidazole was purchased from Sigma Chemical Co. (St. Louis, MO.). All of the other compounds tested in these studies were gifts from Syntex (Palo Alto, CA.) Other reagents were laboratory grade purchased from Sigma unless specifically noted. The antifungals were solubilized in normal saline with the addition of 0.1 N HCl as needed. The pH of these solutions was adjusted to 4.0 with 0.1 N NaOH.

The C. albicans used in most experiments is a clinical isolate previously denoted stn-1 (8). Two isolates of Candida tropicalis, djs-1 and das-1, previously described (7), were also studied. Cultures were periodically examined to insure that there were no contaminating organisms. For typical binding studies, 12 agar plates (Nutrient Agar, Difco, Detroit, MI) were streaked for heavy growth and cultured overnight at 37°C before harvesting.

The [^{3}H]corticosterone binding assay has been described in detail elsewhere (6). Briefly, yeast were harvested from agar plates by scraping and washed twice with 0.9% saline. The cells were broken by vigorous agitation on a vortex mixer with 250-300 µm glass beads. The homogenization buffer consisted of 250 mM sucrose, 10 mM Tris-HCl, 1.5 mM EDTA 12 mM monothioglycerol, and 10 mM sodium molybdate, pH 7.8. Cytosol was prepared by ultracentrifugation at 204,000 x g for 30 min. Aliquots of cytosol (0.25 to 0.5 mg protein/ml) were incubated with [^{3}H]corticosterone ± the indicated competitor for 3 hr at 0°C, conditions previously shown to achieve equilibrium. Bound [^{3}H]corticosterone was separated from free hormone by gel-exclusion chromatography using columns made with G-50 fine Sephadex (Pharmacia, Piscataway, N.J.). Non-specific [^{3}H]corticosterone binding was assessed in all experiments by replicate incubations containing a 250-fold molar excess of radioinert corticosterone. All results were corrected for non-specific binding which was typically 10-15%. Cytosol protein concentration was assessed by the method of Bradford (9), using a mixture of 80% human gamma globulin and 20% bovine serum albumin as standard.

Minimum inhibitory concentrations (MIC) were determined by methods previously detailed (10). The drugs were solubilized in methanol to yield 10 mg/ml stock solutions, except for econazole and miconazole, which were 10 mg/ml stock solutions in crenophor for intravenous use and RS49910 and RS36745, which were 1.58 mg/ml solutions in saline, pH 4.0. These diluents had no antifungal activity at the same concentration present in 100 µg/ml of drug in medium, which was the highest concentration tested in the MIC experiments.

Results

The ability of ketoconazole and several other imidazole antifungals to displace [^{3}H]corticosterone from Candida CBP binding sites was evaluated, and the results are shown in Figure 1. Each of the drugs tested showed a dose-dependent inhibition of [^{3}H]corticosterone binding. Ketoconazole was by far the most potent competitor, inhibiting [^{3}H]corticosterone binding by 50% at a concentration of 0.25 μM (approximately 0.1 μg/ml). Miconazole and econazole were considerably weaker competitors. Miconazole, generally considered equipotent with ketoconazole in in vitro antifungal assays (11), inhibited binding by 50% at about 17 μM. Econazole achieved 50% competition at about 30 μM. Neither clotrimazole nor RS49910 inhibited binding by 50% at concentrations up to 100 μM. We do not understand the mechanism by which clotrimazole increased [^{3}H]corticosterone binding at high concentrations of drug.

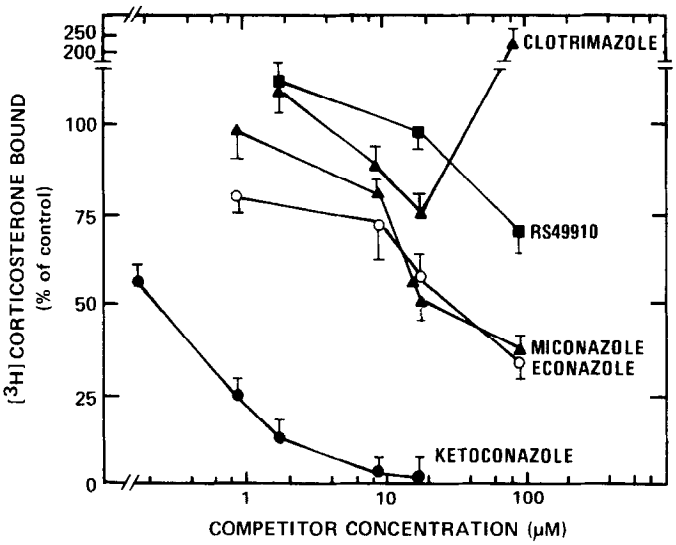

Fig.1 [^{3}H]Corticosterone displacement potency from Candida CBP by imidazole antifungal drugs. Candida cytosol was incubated at 0°C for 3 hours with 13 nM [^{3}H]corticosterone ± the indicated concentrations of antifungal competitors. Protein bound hormone was determined by chromatography over G-50 Sephadex columns. Specific binding in the absence of competitors averaged 620 ± 85 fmol/mg protein and was taken as 100% binding. Each point is the mean ± SEM of at least four determinations. RS 49910 is an aryloxy-substituted alkyl imidazole.

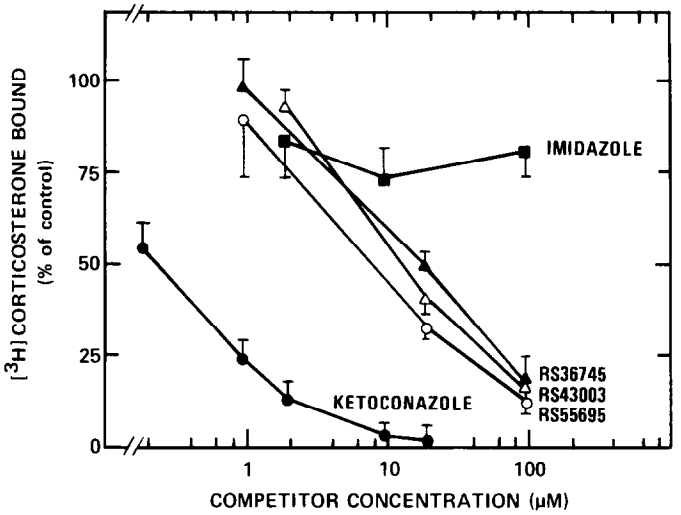

Fig.2 [^{3}H]Corticosterone displacement potency from Candida CBP by imidazole structural analogues. The conditions were the same as described in Fig. 1. Each point is the mean ± SEM of at least four determinations. RS36745 is the trans-isomer of ketoconazole. RS43003 is an alkyl imidazole with molecular formula $CH_3(CH_2)_9N_2C_3H_3$. RS55695 is a ketoconazole analog retaining the entire piperazine side-chain.

Figure 2 illustrates similar competition experiments employing structural analogs of ketoconazole. RS 36745, the trans-isomer of ketoconazole, was substantially less active than ketoconazole as an antifungal in MIC experiments; it was about 1% as potent as the active cis-isomer in its ability to displace [^{3}H]corticosterone from CBP. RS 43003, an alkyl imidazole, has little or no antifungal activity and inhibited [^{3}H]corticosterone binding by 50% at about 20 μM. RS 55695 is a analog of ketoconazole, retaining the entire piperazine side-chain. The structural modification reduced the binding potency by about 50-fold relative to ketoconazole. Imidazole itself was unable to displace more than 25% of the radioprobe at concentrations up to 100 μM.

The nature of the ketoconazole inhibition of [^{3}H]corticosterone binding was examined further by Lineweaver-Burk analysis. C. albicans cytosol was incubated with multiple concentrations of [^{3}H]corticosterone ± 0.2 μM ketoconazole. Double-reciprocal plots of the binding data, shown in Figure 3, indicate that the inhibition of [^{3}H]corticosterone binding by CBP was competitive in nature.

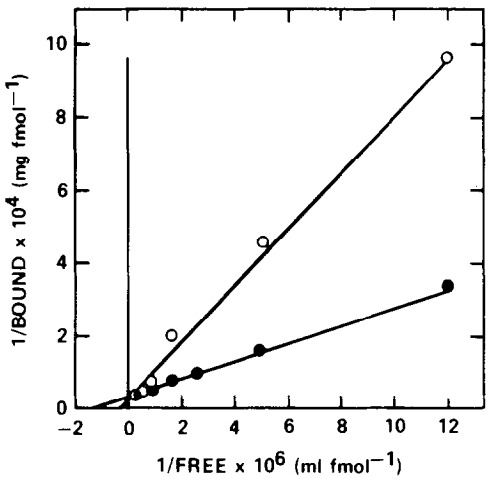

Fig.3 Lineweaver-Burk analysis of ketoconazole competition for Candida CBP binding sites. Candida cytosol was incubated with multiple concentrations of [^{3}H]corticosterone in the absence (0) or the presence (0) of 0.2 µM ketoconazole. Specific binding was determined by Sephadex chromatography.

In additional experiments, ketoconazole displaced [^{3}H]corticosterone from CBP in whole-cell binding assays as well. [^{3}H]corticosterone uptake experiments at 37°C for 30 min demonstrated saturable binding at about 13-26 nM. In good agreement with cytosol binding assays, 0.2 µM ketoconazole competed for about 40% of the specific [^{3}H]corticosterone binding sites, and 2 µM ketoconazole competed for about 70% of the CBP sites. Thus both corticosterone and ketoconazole appear capable of entering the cell, and ketoconazole can displace [^{3}H]corticosterone from CBP in the intact organism.

There was no apparent correlation between binding potency and antifungal potency among the various drugs tested either based upon previously published relative potency data (11,12) or MIC values obtained with the stn-1 isolate (data not shown). We further explored the possibility that binding to CBP was important for ketoconazole action by examining two isolates of Candida tropicalis, one that contained CBP (das-1) and one that demonstrated no specific binding of [^{3}H]corticosterone (djs-1). The MIC data revealed no difference in the antifungal potency of ketoconazole in these two strains; the MIC for both strains was identical (1.6 µM ketoconazole).

Discussion

This paper describes the ability of imidazole antifungal agents to compete with [^{3}H]corticosterone for CBP binding sites in the cytosol of C. albicans. Ketoconazole, a very effective antifungal agent, was at least 50 to 100 times more potent than other imidazole compounds in its ability to displace [^{3}H]corticosterone from CBP (Fig. 1 and 2). This binding was dose-dependent and, at least in the case of ketoconazole, was competitive in nature (Fig.3).

Ketoconazole displaced 50% of the [^{3}H]corticosterone at 0.25 μM, a concentration easily achieved in vivo (13) and clearly within the MIC range of this drug (12). However, the overall ability of the imidazole drugs to compete for CBP did not correlate with their antifungal activity. Miconazole, a potent antifungal (11), was only about 1% as effective as ketoconazole in displacing [^{3}H]corticosterone from CBP. Clotrimazole, equipotent with ketoconazole against the stn-1 isolate of C. albicans (data not shown), failed to displace more than 25% of the binding at concentrations up to 100 μM.

Studies with two isolates of C. tropicalis, one positive and the other negative for [^{3}H]corticosterone binding, showed them to be equally sensitive to the in vitro antifungal actions of ketoconazole. Furthermore, we have previously determined that neither Saccharomyces cerevisiae (14) nor Paracoccidioides brasiliensis (15) possess a CBP, yet both are quite sensitive to the antifungal actions of ketoconazole (ref. 11 and D.A. Stevens, unpublished data).

These observations seem to make it quite clear that ketoconazole binding to Candida CBP is not critical for the drug to exhibit antifungal activity in vitro. Since Candida produces a molecule which binds to CBP (5), we have postulated that this protein may represent a Candida hormone receptor. If so, it is our expectation that CBP mediates some physiological actions in Candida; ketoconazole binding to CBP would thus be expected to modify this hormonal action. Whether this activity subtly enhances the antifungal properties of ketoconazole in vivo remains to be tested.

The structural requirements of the Candida CBP binding site are of interest. Steroids that exhibited high binding affinity for CBP included corticosterone,

progesterone and deoxycorticosterone, but not estrogens, androgens or calciferols (6). Imidazole antifungals as a class were able to compete for the binding site with ketoconazole being the most potent of the drugs tested. Imidazole itself was relatively inactive. Addition of a simple alkyl chain to an imidazole ring (RS 43003) greatly increased binding activity and may be related to increased lipophilicity. On the other hand, trans-ketoconazole (RS 36745) and the ketoconazole analog (RS55695) remain 50 to 100 times less active in binding than ketoconazole. The data indicate a high degree of selectivity and stereospecificity as determinants of binding site activity. It is of interest that despite its weak activity against Candida CBP, clotrimazole is a better competitor than ketoconazole for mammalian glucocorticoid receptors (8). Of the drugs tested, RS49910 was the weakest competitor for glucocorticoid receptor binding sites (8) and was similarly weak in ability to displace [^{3}H]corticosterone from Candida CBP.

In conclusion, we believe that the binding of ketoconazole and other imidazole compounds to Candida CBP is probably unrelated to the antifungal actions of these drugs. The possible functional responses that follow corticosterone or ketoconazole binding to CBP are currently under study. As a potent competitor for Candida CBP binding sites, ketoconazole may prove to be a useful probe in studying hormone action in fungi.

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