Ketoconazole Binds to Glucocorticoid Receptors and Exhibits Glucocorticoid Antagonist Activity in Cultured Cells

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ABSTRACT We have recently found that ketoconazole inhibits adrenal steroidogenesis; in this paper we investigated whether imidazole antimycotic drugs additionally interact with glucocorticoid receptor sites in target tissues. Our approach was to assess the ability of three drugs: ketoconazole, clotrimazole, and RS 49910, to inhibit [^{3}H]dexamethasone binding to hepatoma tissue culture (HTC) cell cytosol. The results indicated dose-dependent, competitive displacement of [^{3}H]dexamethasone binding that was in the potency sequence: clotrimazole > ketoconazole > RS 49910. We then examined the functional response of this binding by measuring tyrosine aminotransferase (TAT) activity in HTC cells. The antimycotics did not exhibit TAT agonist activity and inhibition of basal enzyme levels was not detected. However, the drugs were potent antagonists of dexamethasone-induced TAT activity and the effect was temporally reversible. This antagonist activity was in the same sequence and closely correlated with the binding potency of the three drugs. We conclude that ketoconazole and other imidazole antimycotic drugs possess glucocorticoid antagonist activity by virtue of occupancy of glucocorticoid receptor sites in target tissues.

INTRODUCTION

Ketoconazole is an important new antifungal agent because of its effectiveness against a wide variety of pathogenic fungi after oral administration and its limited number of side effects (1). However, recent investigations in this and other laboratories have demonstrated that ketoconazole is a potent inhibitor of steroidogenesis both in volunteers and in in vitro studies with adrenal (2, 3) and testicular cells (4). Because other drugs known to inhibit steroidogenesis (5) also compete for specific steroid receptor binding sites in target tissues (6), we proceeded to examine the possibility that ketoconazole might additionally interact with glucocorticoid receptors. Since numerous other imidazole antimycotic drugs are available or are in various stages of development (1), we also examined two additional structurally related drugs to ascertain whether any activity found for ketoconazole was specific to that molecule or represented more general properties associated with the imidazole antifungal drugs as a family. Two sets of experiments were undertaken. First, the antifungal agents were tested for their ability to compete with [^{3}H]dexamethasone for glucocorticoid receptor binding sites in cytosol prepared from hepatoma tissue culture (HTC)[1] cells, a classical glucocorticoid target system (7, 8). Second, the antifungal drugs were tested in HTC cells for their ability to either induce or inhibit the glucocorticoid induction of the enzyme tyrosine aminotransferase (TAT; 7, 8). The results of these studies indicate that ketoconazole, and the other related antifungal agents, competitively inhibit [^{3}H]dexamethasone binding to HTC cell glucocorticoid receptors and effectively block dexamethasone induction of TAT. These findings raise the possibility that this class of drugs may prove useful as glucocorticoid antagonists.

METHODS

Cells and tissue culture. HTC cells were routinely grown in T75 flasks in Dulbecco's Modified Eagle's Medium sup-

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[1] *Abbreviations used in this paper:* HTC, hepatoma tissue culture; TAT, tyrosine aminotransferase.

plemented with 5% newborn calf serum, 5% fetal calf serum (all from Gibco, Grand Island, NY), and 1 g/liter glucose. For binding studies, cells were grown for 7–8 d (confluence) with a medium change on days 2 and 5. For enzyme assays, cells were grown for 4–5 d, with serum-free medium used for the last 24 h.

Binding assays. Cytosol binding studies were performed using previously reported techniques (9). Briefly, cells were harvested by scraping and lysed by sonication at 0°C in a medium containing 250 mM sucrose, 1.5 mM EDTA, 10 mM Tris, 12 mM monothioglycerol, and 10 mM sodium molybdate, pH 7.8. Cytosol was prepared by centrifugation at 204,000 *g* for 30 min. Glucocorticoid receptors were assayed by incubating aliquots of cytosol with [^{3}H]dexamethasone (26 Ci/mmol, Amersham Corp., Arlington Heights, IL) with or without competitors for 3 h at 0°C. A correction for nonspecific binding was made in all experiments by subtracting that binding resistant to a 250-fold molar excess of radioinert dexamethasone. Bound hormone was separated from free hormone using mini-columns made with G-50 fine Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ; 9).

Tyrosine aminotransferase activity. TAT activity was assessed by a spectrophotometric method (7, 8). Cells were treated with various antifungal agents with or without dexamethasone for 20 h, lysed by sonication, and aliquots of the 8,000-*g* supernatant were assayed. Soluble protein was determined by the method of Bradford (10).

Drugs. Ketoconazole was obtained from Janssen (Beerse, Belgium). Clotrimazole (11), a topical antifungal developed by Bayer, and RS 49910, an aryloxy-substituted alkylimidazole (Syntex experimental antifungal), were both obtained from Syntex (Palo Alto, CA). Since circulating drug levels are usually expressed on the basis of microgram per milliliter and all of our studies are based on molar concentrations, we note here that 1.88 μM ketoconazole is equivalent to 1 μg/ml.

RESULTS

Ability of antimycotics to compete for receptor binding sites. Our first experiments were designed to ascertain whether antifungal drugs competed with [^{3}H]dexamethasone for glucocorticoid receptor binding sites. As shown in Fig. 1, each of the three drugs inhibited [^{3}H]dexamethasone binding to HTC cell cytosol, but with a widely differing spectrum of activity. The order of potency and the concentration inhibiting 50% [^{3}H]dexamethasone binding was: clotrimazole (0.7 μM or 0.24 μg/ml) > ketoconazole (20 μM or 10 μg/ml) > RS-49910 (50 μM or 21 μg/ml). To put these inhibitory concentrations into perspective, it should be noted that patients being treated with 200 or 400 mg of ketoconazole per day achieve peak levels between 2 and 20 μg/ml (12).

The decrease in [^{3}H]dexamethasone binding seen with the antimycotic drugs was suggestive of competition at the glucocorticoid receptor site. Three additional studies were performed to evaluate this interpretation of the findings (data not shown). First, ketoconazole was examined in competition experiments

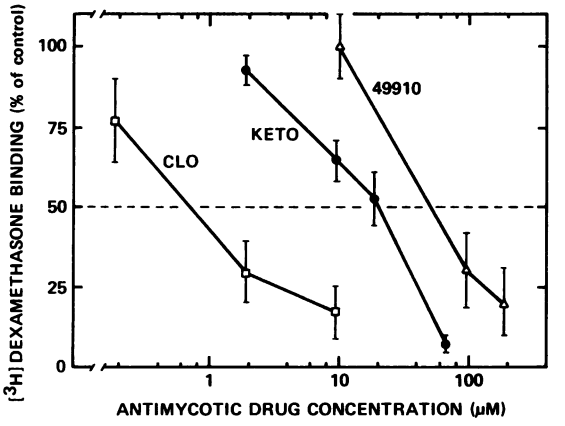

FIGURE 1 Antifungal drug competition for specific [^{3}H]dexamethasone binding sites in HTC cell cytosol. Cytosol was incubated with 13 nM [^{3}H]dexamethasone with or without the indicated concentration of competitors for 3 h at 0°C. Results are expressed as a percentage of control binding obtained in the absence of competitors (109±14 fmol/mg protein). Values shown are mean±SE of four to six determinations. CLO, clotrimazole; KETO, ketoconazole, 49910, Syntex drug RS 49910.

against [^{3}H]estradiol binding to estrogen receptors in rat uterus (13) and against [^{3}H]1,25(OH)$_2$vitamin D_3 binding to vitamin D receptors in rat intestine (14). In both cases, 10 μg/ml of ketoconazole failed to significantly compete for the binding sites indicating that the action against the glucocorticoid receptor was receptor-specific. Second, Lineweaver-Burk analysis of the ketoconazole competition for the glucocorticoid receptor revealed that the inhibition of binding was competitive in nature. Third, these inhibitors were also examined against glucocorticoid receptors freshly prepared from either kidney or thymus cytosol obtained from adrenalectomized rats (9). The results were essentially the same as with HTC cell cytosol.

Effect of antimycotic drugs on TAT induction. Occupancy of the glucocorticoid receptor may result in either the expression of a glucocorticoid-induced function (agonist activity) or blockade of a function induced by glucocorticoids (antagonist activity). To determine which of these possibilities resulted from antimycotic binding to glucocorticoid receptor sites, we examined the effects of these drugs on the induction of TAT activity. Table I shows studies designed to measure agonist activity of the antifungal drugs. Under the conditions examined, these HTC cells exhibited an approximate threefold rise in TAT activity after addition of 13 nM dexamethasone and an approximate sixfold rise after addition of 130 nM dexamethasone. Using concentrations of antimycotic drugs that achieved 50% competition or more for the binding site

TABLE I
Failure of Antimycotic Drugs to Induce TAT Activity

Drug (concentration)	TAT activity
	mU/mg protein
Basal	0.9±0.09
Dexamethasone (13 nM)	2.5±0.33
(130 nM)	5.3±0.58
Clotrimazole (9.4 μM)	0.9±0.03
Ketoconazole (18.8 μM)	1.0±0.18
RS 49910 (65.8 μM)	0.9±0.07

HTC cells were treated with vehicle (basal), dexamethasone, or various antimycotic drugs at the indicated concentrations for 16 h in serum-free culture medium and then TAT activity was measured. Values are mean±SE, n = four to six determinations.

(Fig. 1), none of the three drugs induced a rise in TAT activity above basal levels, indicating the absence of agonist activity.

Fig. 2 shows studies designed to detect antagonist activity. Each of the drugs tested showed a dose-dependent ability to inhibit the dexamethasone (13 nM) induction of TAT activity. Note that the sequence of drug potencies in the binding experiments and the antagonist experiments is the same and that the concentration of drug required to block 50% of the induced TAT (clotrimazole, 1.5 μM; ketoconazole, 12 μM; RS 49910; 82 μM) was roughly equivalent to the concentration required to achieve 50% inhibition of dexamethasone binding (Fig. 1). We do not understand the mechanism by which low doses of RS 49910 increased glucocorticoid-induced TAT activity.

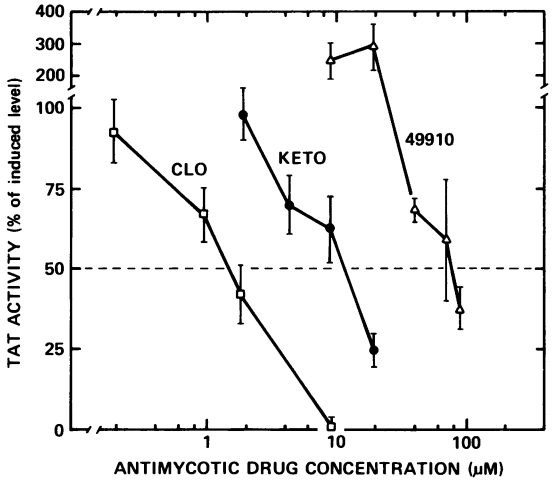

FIGURE 2 Determination of antagonist activity of antimycotic drugs by assessment of effects on glucocorticoid-induced TAT activity. HTC cells were grown for 16 h in serum-free medium in the presence of 13 nM dexamethasone plus the indicated concentration of various antimycotic drugs. TAT induced by dexamethasone above basal is considered 100% and averaged 2.7±0.33 mU/mg protein. Values are expressed as a percent inhibition of the dexamethasone induced TAT activity. Each point is the mean of four to six determinations. CLO, clotrimazole; KETO, ketoconazole; 49910, Syntex drug RS 49910.

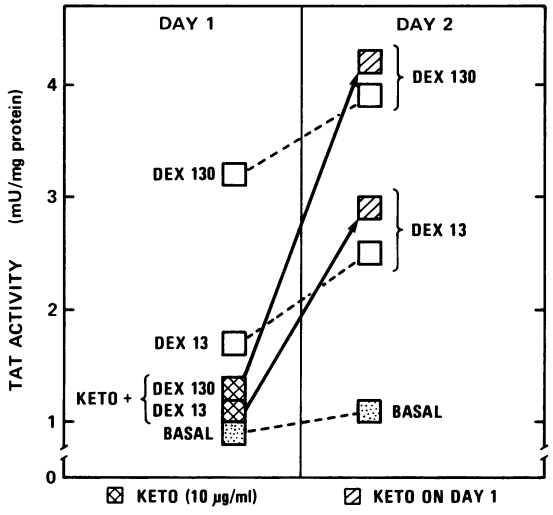

FIGURE 3 Reversibility of the antagonist effects of ketoconazole on TAT activity. Half of a set of flasks of HTC cells was placed in serum-free medium and treated with 18.9 μM (~10 μg/ml) ketoconazole with or without dexamethasone at 13 or 130 nM. After 16 h of incubation (day 1) some cells were assayed for TAT activity. The remaining flasks had a medium change to serum-free medium which also removed ketoconazole from those cells that were treated on day 1. Dexamethasone or vehicle was added for an additional 16 h and then TAT activity was assessed (day 2). DEX, dexamethasone; KETO, ketoconazole.

The antimycotic drugs tested in this study may be cytotoxic at high concentrations. To exclude the possibility that the inhibition of TAT induction was due to such toxic effects, we tested the temporal reversibility of the inhibition of the enzyme by ketoconazole. As shown in Fig. 3, treatment of the HTC cells for 16 h with 10 μg/ml ketoconazole caused a substantial reduction in dexamethasone-induced TAT activity. Cells incubated in parallel for 16 h were then removed from ketoconazole and treated for an additional 16 h with dexamethasone. In this experiment the TAT rise was somewhat greater on day 2 than on day 1 for all cells (treated and control). As shown in Fig. 3 (right panel) the cells pretreated with ketoconazole on day 1 respond to dexamethasone on day 2 to virtually the same degree as the cells treated with dexamethasone on day 2 with no prior ketoconazole treatment.

DISCUSSION

The data presented in this report indicate that imidazole antifungal drugs have the capacity to compete with [^{3}H]dexamethasone for glucocorticoid receptor binding sites. This activity is dose-responsive, competitive in nature, affects glucocorticoid receptors from at least three organs (liver, kidney, and thymus) so it appears generalized, yet is not present for other steroid receptor sites (estrogen and 1,25(OH)$_2$vitamin D). The functional response to the antimycotics is pure glucocorticoid antagonist activity, at least with respect to TAT induction in HTC cells (Table I and Fig. 2). Moreover, the concentration required for binding (Fig. 1) is roughly equivalent to the concentration required for antagonist activity (Fig. 2). Further support for the view that the antagonistic effect is a result of receptor occupancy derives from the findings that the three different drugs tested all caused inhibition of TAT induction with the same relative potency as they competed for [^{3}H]dexamethasone binding sites. The antimycotic drugs did not inhibit basal TAT synthesis expressed either per milligram protein (Table I) or per microgram DNA (data not shown). Increasing the dexamethasone concentration with ketoconazole constant resulted in elevated TAT activity. Furthermore, the antagonist effect was reversed after ketoconazole was removed from the medium (Fig. 3). These findings also provide additional support for the view that the antagonism was not the result of a toxic effect. We conclude from these data that ketoconazole and other imidazole antifungal drugs are glucocorticoid antagonists that act at the level of the hormone receptor. Other known selective corticosteroid receptor antagonists are almost all steroidal in structure (15).

In the light of these findings it is reasonable to ask why patients being treated with ketoconazole do not develop signs of adrenal insufficiency. The drug has most frequently been used in low doses (200 mg/d) and administered only once a day, so that the receptor blockade is likely to be incomplete. Furthermore, because of the pharmacokinetics of the drug, antagonist activity dependent on peak concentrations would not be in effect through much of the day, allowing restoration of hormone action. Protein binding of the drug in plasma might diminish its accessibility to intracellular sites, however ketoconazole does not compete with [^{3}H]corticosterone binding to corticosteroid binding globulin (data not shown). Finally, the syndrome of partial adrenal insufficiency may go unrecognized. From the data presented here, our expectation is that if ketoconazole is used in higher doses or administered more frequently, clinical adrenal insufficiency will likely become apparent.

This phenomenon represents another example of a drug that inadvertently gains occupancy to a "specific" receptor site for a hormone. Within the family of imidazole antimycotic drugs, individual drugs differ substantially in structural characteristics and they do not have obvious homology to glucocorticoids, at least from examination of their two-dimensional structures. Further structure-activity studies are being performed to determine the critical elements for the binding reaction. The role of the binding, if any, in in vivo antimycotic action remains to be clarified.

In previous studies we and others (2–4) have demonstrated that ketoconazole inhibits steroidogenesis in patients and in isolated cells. An agent possessing both the ability to inhibit glucocorticoid synthesis and to blockade glucocorticoid action at the target organ would likely be a potent hormone antagonist. This property of imidazole antimycotics, or specifically designed analogues, might be exploited in settings where glucocorticoid antagonism would be desirable such as the management of certain types of Cushing's syndrome. In addition, a pure glucocorticoid antagonist would be an effective probe useful in laboratory investigations into the mechanism of glucocorticoid action.

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