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Ketoconazole and Other Imidazole Derivatives as Inhibitors of Steroidogenesis

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Background

IMIDAZOLE antifungal drugs are effective therapeutic agents in the treatment of mycotic infections (1). Because ketoconazole can be administered orally and has a broad spectrum of activity against both superficial and deep mycoses, this imidazole derivative has become widely used throughout the world as an antifungal agent (1, 2). In recent years it as become apparent that ketoconazole has additional potent actions to inhibit steroidogenesis. Much of the discussion to follow will be directed at the specific drug ketoconazole and its ability to suppress the synthesis of steroid hormones. However, other structurally related imidazole antifungal agents share in these antihormonal actions. In addition, other categories of drugs contain imidazole or azole structures, and some element of the hormonal effects to be discussed for ketoconazole may also apply to these drugs as well. Prominent examples are cimetidine, thiobendazole, and metronidazole. The ability to inhibit steroidogenesis is especially relevant for the drug etomidate, an imidazole, intravenous sedative-hypnotic agent used for the induction and/or maintenance of anesthesia. Etomidate closely resembles ketoconazole in its effects on steroidogenesis.

A variety of imidazole analogs are clinically useful antifungal agents (3, 4). The first drugs to be introduced were clotrimazole and miconazole which are used primarily as topical agents (3, 4). Subsequently, ketoconazole, which is orally active, has become the major imidazole antifungal drug for systemic use. Ketoconazole and clotrimazole are shown in Fig. 1. Etomidate and aminoglutethimide, two other drugs which inhibit steroidogenesis, are shown for comparison.

Imidazole actions in fungi

The antimycotic imidazole derivatives are believed to exert their antifungal actions by inhibiting the biosynthesis of ergosterol (3–7). Ergosterol, the major sterol in fungi, differs from cholesterol only by having an extra methyl group at carbon 24 and two additional sites of unsaturation, at carbons 7–8 and 22–23. Like cholesterol in higher organisms, ergosterol is a component of membranes and other cellular organelles in fungi. The proposed site of action of the imidazole antifungal drugs is the conversion of lanosterol to ergosterol by the 14α-demethylase system, a microsomal cytochrome P-450 enzyme. Inhibition of ergosterol synthesis may result in direct cellular damage to the fungus. Alternatively, the drug may indirectly injure the fungal cells by the accumulation of 14α-methyl sterols and their abnormal incorporation into membranes and other critical structures. Other important enzymes in the fungus may also be affected by the imidazoles.

Imidazole action in mammalian cells

Although imidazole antifungal drugs were recognized to act by inhibiting a critical cytochrome P-450 enzyme system in fungi, before 1982 it was not appreciated that these drugs also inhibit P-450 enzymes in the host. Imidazole derivatives were known to inhibit mammalian cytochrome P-450 enzymes at high concentrations (8), but at the doses used clinically, the antifungal agents were thought not to affect mammalian P-450 enzymes (9).

The interactions of ketoconazole and other antimycotic imidazoles with cytochrome P-450 enzymes yields type II binding spectra (*i.e.* a peak at about 430 nm and a trough at about 393 nm) (7). The type II change is thought to be due to the formation of a ferrihemochrome by the binding of the unhindered nitrogen in the imidazole ring to the catalytic heme iron atom of the protoporphyrin in the resting cytochrome P-450. This binding prevents the binding of oxygen to the heme and inhibits the ability of the enzyme to act (7).

Imidazole actions on steroidogenic pathways

Elucidation of the actions of imidazoles on mammalian cytochrome enzymes began with a report by DeFelice *et*

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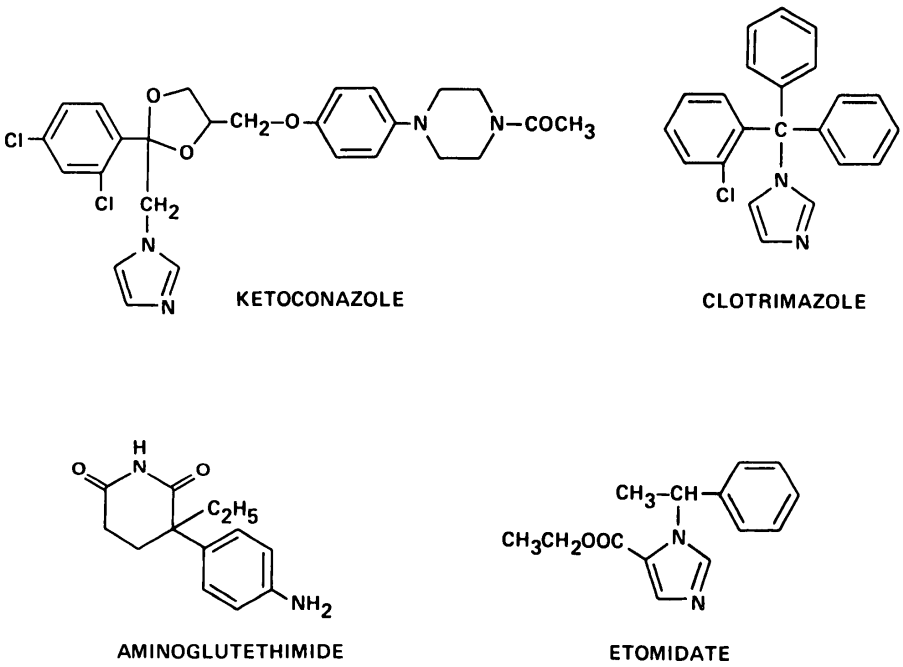

FIG. 1. Chemical structures of cytochrome P-450 enzyme inhibitors.

al. (10) that described the development of gynecomastia in two patients receiving ketoconazole therapy. This led Pont, Stevens, and Feldman (11) at Stanford to investigate the effects of ketoconazole on steroid hormone production. Ketoconazole, administered in therapeutic doses of 200–600 mg/day, lowers circulating testosterone levels in men (11). After a single dose the decline in testosterone concentrations was substantial but transient. Testosterone values return toward normal over 8–24 h as the serum ketoconazole levels fall. Although the fall is dramatic, the transient nature of the decline probably explains why side-effects due to low serum testosterone levels are uncommon with usual antifungal dosages and why the steroidogenic blockade had not previously been noted. This study went on to show inhibition of LH-stimulated testosterone production by rat testicular cells *in vitro*, which demonstrated a direct effect of ketoconazole on the testis (11).

Concurrently, Pont *et al.* (12) similarly demonstrated that ketoconazole in doses of 400–600 mg/day, inhibits adrenal steroidogenesis. Although plasma cortisol levels in volunteers receiving a single dose of ketoconazole were within the normal range, ACTH stimulation tests showed blunted cortisol responses. Furthermore, ketoconazole can block corticosterone production by ACTH-stimulated rat adrenal cells *in vitro*. In this early study we warned the medical community that high or multiple-dose use of ketoconazole might cause hypoadrenalism in patients (12).

These two studies demonstrated direct inhibition of steroidogenesis by ketoconazole in both the adrenal gland and the testis *in vitro* (11, 12). Substantial inhibition was achieved at concentrations of 1–5 μg/ml ketoconazole, whereas peak circulating levels in patients ranged between 2 and 20 μg/ml after conventional doses of 200–400 mg/day (13). These studies raised the possibility that ketoconazole might be clinically useful in patients where inhibition of adrenal and testicular steroidogenesis would be beneficial, specifically in cases of prostatic cancer, hirsutism, and Cushing's syndrome (11, 12). Subsequently, a large number of studies have been performed to elucidate the mechanism of the steroidogenic blockade as well as the clinical utility of ketoconazole as an inhibitor of hormone synthesis.

Ketoconazole Actions on the Adrenal Gland

In vitro blockade of adrenal steroidogenesis

Although in the initial study (12) we demonstrated a direct action of ketoconazole to inhibit ACTH-stimulated corticosterone production *in vitro*, the locus of this action was unclear. In the study by Loose *et al.* (14), we went on to show that ketoconazole inhibits several cytochrome P-450 enzymes. We first showed that ketoconazole did not substantially block ACTH stimulation of cAMP production and that cAMP could not bypass the ketoconazole blockade. Using radiolabeled steroidogenic precursors, the ketoconazole blockade was shown to occur specifically at two enzyme steps, 11β-hydroxylase and cholesterol side-chain cleavage (see Fig. 2 for the adrenal steroidogenic pathway). Both enzymes are mitochondrial P-450 enzymes. Other steroidogenic enzymes

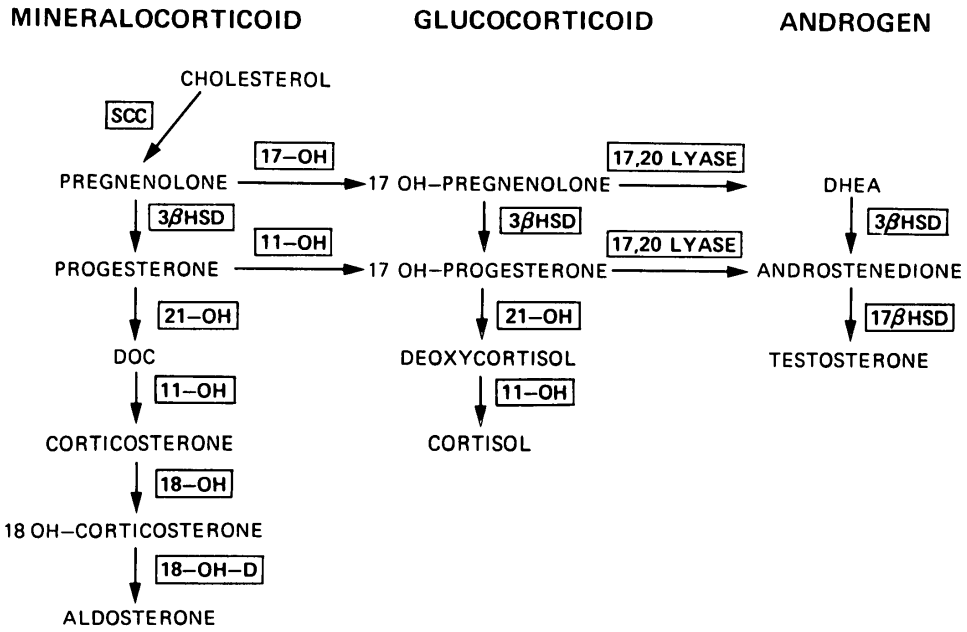

FIG. 2. Pathways of steroidogenesis in the adrenal gland. SCC, Cholesterol side-chain cleavage enzyme; HSD, hydroxy steroid dehydrogenase; OH, hydroxylase; OH-D, hydroxy dehydrogenase.

in the adrenal gland were not inhibited by the doses of ketoconazole tested. We additionally demonstrated that ketoconazole inhibits renal 25-hydroxyvitamin D-24-hydroxylase, another mitochondrial P-450 enzyme, in an extraadrenal tissue. This finding led us to conclude that ketoconazole exhibited an ability for widespread but selective inhibition of P-450 enzymes throughout the body. We predicted the likely inhibition of other P-450 enzymes which might be usefully exploited therapeutically (14).

Kowal (15) also demonstrated inhibition by ketoconazole of 11β-hydroxylase in mouse adrenal tumor cells *in vitro*, and Engelhardt *et al.* (16) showed that ketoconazole could inhibit abnormal cortisol production *in vivo* in a patient with adrenal adenoma. Furthermore, ketoconazole blocked *in vitro* production of cortisol by the excised tumor. Subsequently, Engelhardt *et al.* (17) confirmed that the major locus of ketoconazole inhibition of steroidogenesis in normal subjects and in patients with Cushing's syndrome was 11β-hydroxylase blockade. However, all patients treated with ketoconazole do not exhibit blockade of adrenal steroidogenesis. Dandona *et al.* (18) showed that administration of ketoconazole (400 mg/day) to patients with acute leukemia in the long term prophylaxis of fungal infections did not cause adrenal suppression. In fact, these patients exhibit elevated plasma cortisol levels, presumably related to the stress of the underlying disease.

Imidazole antifungals as glucocorticoid antagonists

At the same time that we were studying ketoconazole as an inhibitor of steroid enzyme synthesis, my coworkers and I were describing a corticosteroid binding protein (CBP) in *Candida albicans* that exhibits high affinity for corticosterone and progesterone (19–21). Since ketoconazole is an inhibitor of ergosterol synthesis in fungi (7), and of steroidogenesis in the mammalian adrenal gland (12, 14), we tried to use ketoconazole to perform "chemical adrenalectomy" in *C. albicans* to prevent the production of putative endogenous fungal hormones (19). These studies gave unexpected results until it became clear that ketoconazole binds to the Candida CBP (22). Ketoconazole is 50–100 times more potent than structurally related imidazoles in displacing [^{3}H] corticosterone from Candida CBP. However, this ability to bind to Candida CBP did not seem to correlate with antifungal potency. The significance of the ability of ketoconazole to bind to Candida CBP remains to be determined (22).

Because of ketoconazole binding to CBP, we then evaluated the ability of ketoconazole to bind to glucocorticoid receptors in mammalian cells (23). Ketoconazole and other imidazole derivatives such as clotrimazole were found to inhibit [^{3}H]dexamethasone binding to glucocorticoid receptors in hepatoma tissue culture (HTC) cell cytosol. When HTC cells were treated with these imidazoles, tyrosine aminotransferase activity is not induced whereas dexamethasone induced a 5-fold increase in this

classical glucocorticoid inducible enzyme. This indicated that, despite binding to the receptor, ketoconazole lacks glucocorticoid agonist activity. However, when ketoconazole is added to HTC cells along with dexamethasone, a dose-dependent, competitive inhibition of tyrosine aminotransferase induction is observed. The glucocorticoid antagonist activity of the imidazoles correlates with binding potency, suggesting that the antifungals possessed glucocorticoid antagonist activity by virtue of their occupancy of glucocorticoid receptors. Antagonist activity was also demonstrated *in vivo* in adrenalectomized rats treated with dexamethasone. This was the first demonstration of a nonsteroidal molecule possessing antiglucocorticoid activity (23). The ability of ketoconazole to inhibit corticosteroid synthesis (12, 14) and to block corticosteroid action at the receptor level, led us to predict that ketoconazole might be an ideal antiglucocorticoid in various clinical disorders of cortisol overproduction (23).

Effect of ketoconazole on circulating corticosteroids in patients

Conventional doses of ketoconazole (200–600 mg/day) cause transient declines in serum testosterone and transient blunting of ACTH stimulation tests (11, 12). Endocrine complications in patients treated with antifungal doses of ketoconazole have not been common. Higher dose regimens (800–1200 mg/day), in patients with more serious fungal infections, cause more prolonged and profound hormone inhibition (24). Urinary free cortisol excretion falls to about 50% of pretreatment levels, ACTH stimulation of cortisol is substantially blocked, and testosterone levels are subnormal around the clock. The side effects of impotence, gynecomastia, decreased libido, and oligo- and azospermia are common at these doses. Thus high dose ketoconazole regimens caused substantial inhibition of testicular and adrenal steroidogenesis.

Clinical trials of ketoconazole in Cushing's syndrome

The efficacy of ketoconazole to inhibit cortisol production *in vitro* and *in vivo* led to its trial as an inhibitor of steroidogenesis in cases of cortisol overproduction. Engelhardt *et al.* (16, 17) described a lowering of plasma cortisol levels in Cushing's syndrome patients and postulated that compensatory rises in ACTH, which were noted, might overcome the steroidogenic blockade during prolonged treatment. It was suggested that ketoconazole might be more effective in cases where the pituitary-adrenal axis had previously been suppressed. This might also be achieved by concomitant administration of dexamethasone and ketoconazole which, in addition, would prevent hypoadrenalism.

Contreras *et al.* (25) successfully treated a woman with Cushing's syndrome due to a functioning adrenal rest tumor of the liver. Clinical improvement was evident early in the course of ketoconazole treatment (400–1000 mg/day) and was associated with striking reduction in serum and urinary sex hormone levels. Cortisol levels remained elevated after clinical improvement was evident, suggesting that glucocorticoid antagonist activity (23) might have contributed to some of the clinical improvement. After normalization of the hypercorticoid state, the patient successfully underwent surgery and achieved an apparent cure.

Five cases of Cushing's disease with recurrent severe hypercortisolism after transsphenoidal hypophysectomy were treated with ketoconazole (400–800 mg/day) by Sonino *et al.* (26). The patients showed immediate decreases in urinary cortisol levels, and clinical features of the disease regressed in all patients. The authors believe that ketoconazole was highly effective in treating their patients, although subsequent pituitary irradiation or bilateral adrenalectomy was undertaken in four of the cases. The patients did not manifest compensatory increases in ACTH levels, possibly because of prior pituitary surgery.

Similar effective results were noted in the report of Angeli and Frairia (27) in which five patients with Cushing's disease were effectively managed with 600–800 mg/day ketoconazole. An intriguing observation in these patients was a fall in ACTH levels along with the plasma cortisol, which is unexplained.

In Cushing's syndrome due to ectopic production of ACTH by inoperable tumors, chemical adrenalectomy is often a therapeutic goal. Shepherd *et al.* (28) reported a case of small cell lung cancer in which chemotherapy was discontinued because of the development of pulmonary infection. Ketoconazole therapy caused a dramatic reduction in cortisol production. Despite continued elevation of ACTH, cortisol levels were controlled. On a dose of 1200 mg/day the patient did well until he experienced rapid tumor progression in lung and liver. The dose of 1200 mg/day caused no side effects. Spironolactone, given to treat hypokalemia, was discontinued. The authors concluded that ketoconazole was a valuable palliative adjunct in malignancies associated with corticosteroid overproduction.

Contreras *et al.* (29) used ketoconazole to treat several cases of Cushing's syndrome due to adrenal tumor. In one female patient with metastatic adrenal carcinoma, the picture of Cushing's syndrome improved, and the tumor regressed (30). On doses of ketoconazole between 800 and 1200 mg/day (with 1.5 mg/day dexamethasone), cortisol overproduction was controlled. Of great interest, x-ray, computed tomography scanning, and ultrasonography demonstrated almost complete disappearance of

lung metastases, decrease of a liver mass, and regression in the size of the primary adrenal tumor. Over the 3 months of observation, the patient showed associated clinical improvement with improved well being, return of appetite, weight gain, and disappearance of jaundice. Since regression of metastatic adrenal tumors is unusual, the authors speculated on whether this adrenal carcinoma might have been a hormone-dependent tumor. Glucocorticoid receptors are known to be present in normal adrenocortical cells (31) and in some adrenal cancer cells (32).

Ketoconazole Actions on the Testis

Blockade of testicular steroidogenesis

The initial study by Pont *et al.* (11) demonstrated a decline in serum testosterone levels in volunteers as well as direct inhibition of LH-stimulated testosterone synthesis by isolated testicular cells *in vitro*. The site of the blockade was not yet elucidated, but steroid patterns in patients taking ketoconazole (decreased androstenedione and increased 17α-hydroxyprogesterone) suggested a blockade of 17,20-lyase (33) (see Fig. 3 for steroidogenesis pathways in the testis). This enzyme complex is a microsomal, cytochrome P-450 enzyme which converts 17α-hydroxyprogesterone to androstenedione. Schürmeyer and Nieschlag (34) demonstrated that the inhibitory effect on testosterone synthesis also occurs with other antifungal imidazole drugs such as miconazole and clotrimizole. Kan *et al.* (35) and Sikka *et al.* (36) subsequently demonstrated direct inhibition of 17,20-lyase activity by ketoconazole in rat testicular cells *in vitro*. Lauwers *et al.* (37) confirmed the presence of 17α,20α-dihydroxyprogesterone accumulation in testicular cells by mass spectroscopy providing supportive evidence for the lyase inhibition. However, in our study (35) we did not observe inhibition of 17α-hydroxylase, even at ketoconazole concentrations up to 50 μM, while cholesterol side-chain cleavage enzyme was also inhibited, as in the adrenal gland (14). Sikka *et al.* (36) did not test for side-chain cleavage inhibition but did detect inhibition of 17α-hydroxylase, in contrast to our findings (35). Since 17,20-lyase and 17α-hydroxylase activity have been found to be present in a single protein (36a), it is of some interest to resolve whether ketoconazole inhibits the two enzyme activities differently. The clinical data clearly indicate more prominent inhibition of lyase activity with elevated 17α-hydroxyprogesterone levels (33).

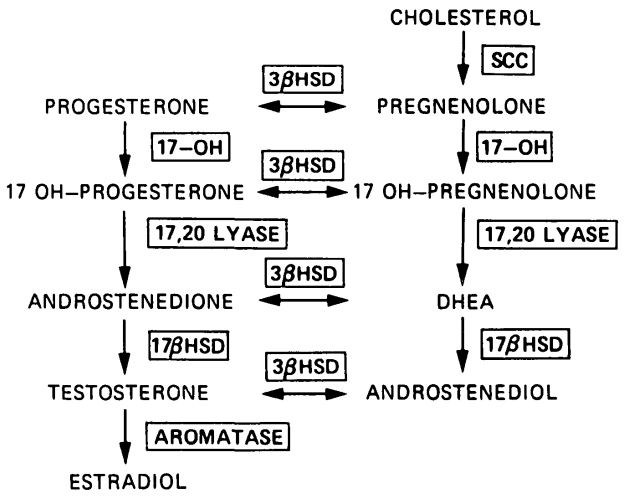

FIG. 3. Pathways of steroidogenesis in the testis. Abbreviations defined in Fig. 2 legend.

In a subsequent study Bhasin *et al.* (38) showed a lack of direct ketoconazole inhibition of gonadotropin release at the pituitary level in rats. In addition to their earlier *in vitro* findings of inhibition of 17,20-lyase and 17α-hydroxylase (36), in this study which was performed after 4 weeks of ketoconazole treatment, they also detected inhibition of 17β-hydroxy steroid dehydrogenase activity. Since this latter steroid is not known to be a cytochrome P-450 enzyme, the inhibition of this enzyme does not fit the established general pattern, and confirmation of direct inhibition of non-P-450 enzymes by ketoconazole is required.

In our study (35), four different imidazoles were compared in their ability to inhibit testosterone synthesis in testis and 25-OH-vitamin D-24-hydroxylase activity in kidney. We were struck by the differences in the pattern of enzyme inhibition and suggested that it might be possible to design more specific imidazole antagonists which might selectively block a specific P-450 enzyme pathway in a given organ at dosages low enough not to block other P-450 enzymes.

Effect of ketoconazole on circulating sex steroids

Single dose ketoconazole treatment (400 mg) of volunteers lowered testosterone levels to 18% of control and estradiol level to 60% of control (39). The nadir was reached 6 h after drug administration, and values returned to normal before 24 h. Pont *et al.* (40) found negligible reduction in estradiol levels, even after 600 mg/day. Several studies have found that free testosterone levels correlate with total testosterone levels (39–41). Higher doses of ketoconazole (800–1200 mg/day), even once daily, cause more pronounced and more prolonged androgen blockade, and in some men testosterone levels are depressed throughout the day (24).

In summary, it appears testosterone is more depressed than estradiol by ketoconazole administration. Since estradiol is produced from testosterone by aromatase enzyme activity, one might expect that this enzyme would not be inhibited or might even be stimulated by ketoconazole. Mason *et al.* (42) studied the ability of imidazole

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