Inhibition of Testosterone Production with Ketoconazole Alone and in Combination with a Gonadotropin Releasing Hormone Analogue in the Rat

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ABSTRACT

Ketoconazole is a well-tolerated, synthetic, imidazole derivative currently in widespread therapeutic use against mycotic infections. Recent evidence that it depresses testosterone synthesis in humans prompted us to investigate the effects in rats of its administration alone or in combination with the gonadotropin releasing hormone superagonist analogue leuprolide. Plasma luteinizing hormone, testosterone, and ketoconazole levels as well as ventral prostate weight and tumor growth in rats bearing the androgen-dependent Dunning R3327H model of prostate adenocarcinoma were measured. Doses of 30 mg/kg twice daily of ketoconazole alone depressed plasma testosterone levels by approximately 75% to a nadir of 0.47 ± 0.08 (SE) ng/ml on day 20 (P < 0.001 versus basal). This effect of ketoconazole was exerted directly at the testicular level since plasma luteinizing hormone levels were not suppressed. In response, ventral prostate weight declined and growth of the Dunning R3327H tumor was retarded to rates observed in castrate controls. Leuprolide alone lowered basal testosterone levels to 0.20 ± 0.02 ng/ml after 35 days of daily administration but persistent androgen increments after each injection (acute-on-chronic effect) were observed (i.e., to 4.41 ± 0.62 ng/ml). The addition of ketoconazole to leuprolide inhibited the acute-on-chronic rise in testosterone to 0.33 ± 0.07 ng/ml and also lowered basal testosterone levels further to 0.11 ± 0.01 on day 10 of combined administration. Ketoconazole also blunted the response to the first injection of leuprolide from a 3-h peak level of 8.74 ± 0.53 to 4.17 ± 0.80 with the 40 mg/kg dose. These results indicate that combining ketoconazole with leuprolide achieves greater suppression of testosterone than either agent alone. When such protocols are applied to humans with prostate cancer, more extensive effects may be expected because of the greater sensitivity of patients than of the rodent species to these agents.

INTRODUCTION

Reduction of testicular androgens by surgical castration or diethylstilbestrol administration induces tumor regression in three-quarters of men initially treated for metastatic prostatic carcinoma (1). While plasma testosterone levels fall 4- to 8-fold, the adrenals continue to secrete testosterone as well as androgenic substrates of androstenedione (2-5). Considerations regarding the adrenal led to the recent hypothesis that complete inhibition of adrenal as well as testicular androgens might be important in the treatment of men with prostatic cancer (6). Clinical trials to test this possibility have utilized antiandrogens as a means of combating the effects of adrenal androgens. With this approach, surgical castration or gonadotropin releasing-hormone superagonist analogues are used to inhibit the testicular androgen source while the antiandrogen combats the effects of persistent androgens arising from the adrenal (6). An alternate strategy is to inhibit directly androgen biosynthesis at both the testicular and adrenal level.

To explore the approach using synthesis inhibitors, we examined the effects of ketoconazole (Nizoral; Janssen Pharmaceuticals-R41400), a blocker of testicular and adrenal androgen biosynthesis (7), alone and in combination with leuprolide (o-leucine-α-gonadotropin-releasing hormone proethylamide; Abbott-43818), an inhibitor of LH secretion (8). The results suggest that the combination inhibits testosterone to a greater extent than either approach used alone in the intact male rat.

MATERIALS AND METHODS

Animals and Tumors. Rats used in this study were Fischer/Copenhagen F1 or Noble males obtained from our breeding colonies maintained in the Department of Comparative Medicine, The Milton S. Hershey Medical Center. The Dunning R3327H tumor was obtained through the generosity of Dr. N. H. Altman (Papanicolaou Cancer Research Institute, Miami, FL). The original tumor was received in a Fischer/Copenhagen F1 male bearing a palpable s.c. tumor which was allowed to grow until a size of 5000 mm³ was reached. At that point, under aseptic conditions, the tumor was excised, debrided of connective tissue, and minced into small fragments (1.5 x 1.5 mm) in a sterile dish containing saline. Recipient F1 males were anesthetized and given implants s.c. in the flank region of one tumor fragment. The animals were allowed to recover, replaced in their cages, and there remained undisturbed until the tumors grew to a size of approximately 5000 mm³. The animals were then randomly allocated into one of the experimental groups.

The tumors were solid and homogeneous with no signs of central necrosis. Histological preparations revealed that they were well differentiated with epithelial-lined acinar structures dispersed in a tissue matrix consisting of fibroblast-like cells.

Drugs. Ketoconazole (R41400) was obtained as a powder from Janssen Pharmaceutical Co. Material for s.c. injection was prepared by dissolving the powder in pure corn oil (Mazola) with rapid stirring or by dissolving in 0.2 N HCl. Leuprolide (Abbott-43818) was obtained as a powder from Abbott Laboratories. Material for s.c. injection was prepared by dissolving the drug in sterile water.

Plasma Measurements. Plasma ketoconazole levels were determined by a modification of the agar diffusion method of Levine and Cobb (9). Testosterone was measured after extraction of 0.2-0.5 ml serum with
0.5 ml methylene chloride. Samples were assayed using immunotestosterone-125I kits supplied by Pantex Corporation (Santa Monica, CA). Dihydrotestosterone cross-reactivity with this antibody is 35%; all other steroids cross-react less than 0.1%. LH was measured by a radioimmunoassay method, as previously published, with reagents supplied by the National Pituitary Agency (8).

Protocol for Studies

Determination of Ketoconazole Dose. In a pilot study, adult Fischer/ Copenhagen F, male rats received ketoconazole (either 10 or 30 mg/kg) prepared in corn oil, by s.c. injection b.i.d. Serum was obtained on days 0 (before initiation of treatment), 3, 7, and 14. The samples on days 3 and 7 were collected 4 h after the 8 a.m. injection and on day 14, 12 h later.

Detailed Hormonal Effects of Ketoconazole. Based upon the data from the pilot study, ketoconazole, 30 mg/kg prepared in corn oil, was injected s.c. b.i.d. to 6 groups of 7 adult male Noble rats each for a period of 20 days. Blood samples were obtained on days 0 (before initiation of treatment), 1, 3, 10, 15, and 20. To reduce variance (10) resulting from pulsatile LH and testosterone secretion (11) as well as diurnal variation (12-14), three blood samples were obtained from each animal. These were collected 2, 4, and 6 h after the 8 a.m. injection of ketoconazole and at the same time points in nontreated controls (day 0 animals). Each of the 3 samples was assayed and the mean was recorded and used to assess drug effects.

Leuprolide Alone. Based upon prior studies (8), a dose of 100 μg of leuprolide was selected and administered by single daily (11 a.m.) s.c. injection to normal intact male rats for a period of 35 days. Blood samples were collected at 0 (before leuprolide injection), 3, 6, and 9 h after the first dose in the acute phase and at 0 and 3 h in the chronic phase.

Leuprolide plus Ketoconazole. In acute experiments, 2 groups of intact adult male Noble rats were given ketoconazole by gavage at a dosage of either 20 or 40 mg/kg, at 0 (10 a.m.), 2, and 4 h. At hour 3 (1 h after the second ketoconazole dose), serum was obtained and 100 μg of leuprolide was administered by s.c. injection. Three h later (2 h after the last ketoconazole dose), serum was again obtained and assayed for testosterone. Animals receiving leuprolide alone served as controls.

For the chronic studies, leuprolide was administered by once daily (11 a.m.) injection for a total period of 45 days. The administration of ketoconazole (prepared in corn oil) was initiated on day 35 at a dosage of 30 mg/kg. Ketoconazole was administered by s.c. b.i.d. injection and continued until day 45. Serum for testosterone assay was obtained immediately before and 3 h after leuprolide injection on day 35 (before initiation of ketoconazole treatment) and on days 38, 42, and 45 of combined leuprolide plus ketoconazole administration.

Protocol for Tumor Growth. Twenty intact, adult, Fischer/Copenhagen F, male rats bearing established Dunning R3327H tumors were paired according to similar tumor size. One member of each pair was assigned to the ketoconazole group and the other to the vehicle group. The ketoconazole group received 30 mg/kg s.c. b.i.d. for 14 days whereas the vehicle group received pure corn oil. Tumor measurements by caliper were performed on day 0 (before initiation of drug treatment) and on days 3, 7, and 14. Tumor volume was approximated from the measurements (15) and the data were expressed as percentage volume change from basal on day 0.

For comparative purposes, an additional 8 intact animals bearing established tumors of similar size to the drug and vehicle animals were castrated via the scrotal route. Tumor measurements were obtained by the above method on days 0 (before castration), 3, 7, and 14.

Statistical Analysis. Paired or unpaired t tests were used for single comparisons between data. For multiple comparisons, the Newman-Keuls analysis of variance or the Bonferroni correction (16) of the unpaired t test was used.

RESULTS

Ketoconazole Alone

Pilot Study. Both drug doses of ketoconazole in corn oil equally suppressed peripheral testosterone levels to a similar extent. The 10 mg/kg b.i.d. dose caused a decrease of 50% by day 3, 78% by day 7, and 65% by day 14. Reductions of 50, 85, and 70% occurred at identical time points in response to the 30 mg/kg dosage. The biological effects of this inhibition in androgen secretion were assessed by quantitating the growth of the androgen-sensitive Dunning R3327H tumor in animals receiving the 30 mg/kg dose. A slight reduction in tumor growth compared to that observed in intact animals was observed by the first wk (Chart 1). Tumors in the ketoconazole-treated animals then decreased in size over the next 7 days and approached tumor volumes observed in animals treated with surgical castration.

Detailed Study. For a more complete evaluation of drug effects, we then utilized the 30 mg/kg b.i.d. dose for a total period of 20 days. In response to ketoconazole, testosterone fell gradually from 1.2 ± 0.08 (SE) ng/ml to a nadir of 0.47 ± 0.08 (P < 0.001) on day 20 (Chart 2, middle). Because of the marked variance of testosterone levels among animals (10) and stringency of the Newman-Keuls test, only the day 20 levels were statistically significantly different (i.e., P < 0.001) from basal. Ventral prostate weights also gradually fell in parallel with the decline in testosterone levels (Chart 2, bottom). The changes in testosterone and prostate weight reflected the peripheral actions of ketoconazole on testosterone biosynthesis rather than a central suppressive effect on LH. As support for this conclusion, no decline but rather a slight (but not statistically significant) rise in plasma LH levels was observed during the protocol (Chart 2, top).

In order to ensure the persistence of adequate plasma ketoconazole levels during the protocol, drug concentrations were measured in plasma pools from 7 rats each at frequent intervals after the first dose (Chart 3, solid line) and then at 6- and 12-h intervals during chronic dosing (Chart 3, dashed lines). Initially levels gradually increased and reached a plateau (10-12 h) at 2.9 μg/ml. During chronic dosing, the levels at 12 h (i.e., just before subsequent dosing) were maintained between 1.2 and...

Chart 1. Mean percentage change in the volumes of the Dunning R3327H tumor in intact (O) and castrate (□) animals and in those receiving ketoconazole, 30 mg/kg b.i.d., by s.c. injection (□). Statistical comparisons are intact versus ketoconazole treated on days 3, 7, and 14 by Newman-Keuls analysis. ***, P < 0.001; bars, SE.
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2.9 μg/ml and somewhat higher at 6 h after dosing (i.e., 2.1–4.2 μg/ml).

Leuprolide Alone and in Combination with Ketoconazole

Acute. To determine the time-related effects of leuprolide, blood samples were collected at intervals for 9 h after a single dose of 100 μg of the superagonist analogue. Peak testosterone levels of 11.8 ± 1.51 ng/ml were observed at 3 h with a fall at 9 h to 4.34 ± 1.29 ng/ml. In a separate experiment, ketoconazole in a series of 3 doses of either 40 or 20 mg/kg was then given by gavage and the 3-h peak testosterone response to leuprolide was again evaluated. Ketoconazole blunted this response from 8.73 ± 0.53 ng/ml (controls, leuprolide only) to 5.39 ± 1.28 and 4.17 ± 0.80 ng/ml in a dose-related fashion.

Chronic. Continued daily administration of 100 μg of leuprolide lowered basal testosterone levels to 0.20 ± 0.02 ng/ml on day 35 (Chart 4). However, as observed by others, an acute effect of each daily dose of leuprolide was observed during chronic therapy (i.e., so-called "acute-on-chronic" effect) (8, 17). For example, the levels of plasma testosterone increased from 0.20 ± 0.02 to 4.41 ± 0.62 ng/ml 3 h after leuprolide on day 35 (Chart 4, open circles). Addition of ketoconazole, 30 mg/kg s.c. b.i.d. during chronic leuprolide treatment, exerted 2 effects. Basal testosterone levels fell to 0.07 ± 0.009 ng/ml after 3 days of combined therapy and to 0.11 ± 0.01 ng/ml after 10 days (Chart 4, inset). In addition, the acute-on-chronic effect of leuprolide was blunted. Testosterone levels measured at 3 h after the leuprolide injection decreased from 4.41 ± 0.62 ng/ml (leuprolide alone) to 3.73 ± 0.52 ng/ml (leuprolide plus ketoconazole) during combined therapy.
alone, day 35) to 0.33 ± 0.07 on day 10 of combined administration.

**DISCUSSION**

In men, 95% of the circulating androgen pool arises from testicular secretion (4). Another 3-5% results from direct adrenal secretion of testosterone or of androstenedione which is then converted in peripheral tissues into testosterone (4, 6, 18, 19). The dog and rat adrenal also secretes significant amounts of testosterone, dihydrotestosterone, and androstenedione (20, 21); however, direct data from other species are not available. Certain clinical observations also suggest that the adrenal androgens may be important in men with prostate cancer. After surgical castration, the absolute levels of serum testosterone are decreased 4- to 8-fold and although reported values vary, persist in the range from 28 to 137 ng/100 ml (2-5). Similarly surgical castration decreases the levels of dihydrotestosterone in prostate cancer tissue by 30 to 50% but these remain surprisingly high (i.e., 1.2–2.3 ng/g) (22). Surgical or medical adrenalectomy produces objective tumor regression in 10–15% of castrate men with prostate cancer and objective stabilization in another 20–30% (18, 23, 24).

Based upon these observations, we considered it pertinent to explore methods of blocking androgen secretion by both the testes and adrenals in an animal model. Prior studies from our and other groups demonstrated inhibition of testicular androgens in rats and humans by administration of gonadotropin-releasing hormone superagonist analogues (25, 26). We and others also used ketoconazole in men and found an inhibition of testosterone through blockade of the C17-20 lyase step (7, 27–29). This reaction occurs both in the testes and adrenals and ketoconazole has been shown to block androgen biosynthesis in both organs. Our experience with both drugs prompted a systematic study of each alone and in combination in the rat as an experimental model.

Ketoconazole produced significant but incomplete inhibition of androgen biosynthesis when given alone. Larger doses or alternate routes of administration might produce more sustained and higher blood levels of drug, thus lowering testosterone levels further. In vitro, ketoconazole concentrations greater than 5 µg/ml completely inhibits testicular and adrenal androgen biosynthesis (28, 30–32). In our study, plasma concentrations of less than 5 µg/ml (i.e., 2.5–4.2 µg/ml) of ketoconazole were usually observed. The study by Trachtenberg (33) in rats demonstrated greater inhibition of testosterone when 10 mg ketoconazole/animal were given chronically by i.p. injection. The greater suppression may have reflected higher ketoconazole plasma levels achieved by using this route of administration. Unfortunately plasma levels of ketoconazole were not measured in this study to allow comparison with our data.

The observations in the present study are consistent with those in men regarding the efficacy of ketoconazole used alone. Testosterone levels fall to 4.8 ± 0.8 nmol/liter (138.2 ± 23 ng/dl) in patients with prostate cancer who are given maximally tolerated doses of ketoconazole (400 mg) 3 times daily (34–36). These levels are higher than observed in castrate (11.0 ± 0.9 ng/dl) or leuprolide-treated men (19.0 ± 4.4 ng/dl) (9) or in castrate men treated with a medical adrenalectomy regimen (12.0 ± 2.0 ng/dl) (24). Taken together, these data suggest that ketoconazole alone at the maximally tolerated dose cannot completely inhibit testicular and adrenal androgen output.

Leuprolide alone inhibits androgen secretion incompletely in the rat since each acute dose during chronic therapy induces a pulse of testosterone secretion by the testis. Because of this, the ventral prostate weight during treatment never involves to the extent seen with castration (8). In men, leuprolide inhibits testicular androgen production into the castrate range but adrenal secretion persists (8). Thus in men and in rats, both suppressive regimes (i.e., ketoconazole or leuprolide) incompletely inhibit androgen production when used alone.

The combination of ketoconazole and leuprolide in rats more effectively inhibited androgen production than did either when given alone. Basal testosterone levels fell from 0.20 ± 0.02 to 0.11 ± 0.01 ng/ml when ketoconazole was added to chronically administered (i.e., 35 days) leuprolide. In addition, ketoconazole blunted the acute-on-chronic testosterone rise after each dose of leuprolide as well as the initial (acute) testosterone rise in response to leuprolide. Nonetheless testosterone levels remained measurable during combined therapy in the rat.

How do the current data relate to the ultimate application of combined ketoconazole and leuprolide therapy to patients with prostate cancer? It must be recognized that the rat when compared to man is less sensitive to the effects of both leuprolide and ketoconazole (6, 8, 35). Castrate levels of testosterone are routinely observed in men treated with leuprolide and acute-on-chronic testosterone pulses do not occur (8). Six-fold decrements of testosterone occur in men treated with ketoconazole versus only a 3-fold suppression in rats (33–35). Consequently the combination of ketoconazole and leuprolide would be expected to lower testosterone more effectively in men than in rats. Indeed preliminary data from Allen et al. (37, 38) suggest that lower than castrate levels of testosterone can be achieved in men with prostate cancer given ketoconazole after exposure to chronic leuprolide.

Two potential strategies could be used to achieve complete androgen ablation. The first utilizes an antiandrogen to block the effects of residual adrenal androgen production in castrate or gonadotropin-releasing hormone agonist-treated men. The second blocks androgen biosynthesis completely by inhibiting both testicular and adrenal secretion. It is pertinent to consider under what circumstances the inhibitors of biosynthesis would be preferable to the use of antiandrogens. A recent strategy for treatment of prostate cancer uses a protocol of androgen depletion followed by acute repletion to partially synchronize cancer cells in the S phase of cellular division and thereby to potentiate the effects of cytotoxic chemotherapy (39). Partial synchronization has been demonstrated in the Dunning rat prostatic cancer model and preliminary data in men indicate the feasibility of such a treatment strategy. The conceptual principle underlying this approach is that acute androgen repletion will immediately stimulate endocrine-sensitive cancer cells to undergo mitosis. Antiandrogen administration confounds the ability of cancer cells to respond immediately to androgen administration. Clearance of the antiandrogen from plasma and favorable stoichiometric competition between drug and androgen is necessary before full expression of the effects of androgen administration is possible.

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Strategies using inhibitors of androgen biosynthesis in contrast allow immediate responses to administered androgen. For this reason, complete androgen inhibition with biosynthesis inhibitors is preferable when androgen depletion-repletion strategies are being tested.

Prior studies utilized aminogluthethimide as the means to inhibit adrenal androgen biosynthesis (24). We consider that ketoconazole may be preferable since it blocks testosterone at a more distal synthetic step. Ketoconazole inhibits C17-20 lyase, a step which blocks conversion of 17α-hydroxyprogesterone to androsterone (7). In contrast, aminoglutethimide inhibits cholesterol side chain cleavage, a more proximate step in testosterone biosynthesis. For this reason, ketoconazole is probably more potent and more specific as an inhibitor of testosterone biosynthesis.

In summary, ketoconazole or leuprolide produce incomplete androgen blockade in the rat when given alone. In combination, these drugs block androgen secretion more effectively but still not completely. However, because men are more sensitive to both compounds, further exploration of the combination of these 2 drugs in patients remains necessary.

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REFERENCES


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