Ketoconazole Binds to the Human Androgen Receptor

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Summary

Ketoconazole, an imidazole anti-fungal agent, has often produced features of androgen deficiency including decreased libido, gynecomastia, impotence, oligospermia, and decreased testosterone levels, in men being treated for chronic mycotic infections. Based on these potent effects on gonadal function *in vivo* as well as previous work *in vitro* demonstrating affinity of ketoconazole for receptor proteins for glucocorticoids and 1,25(OH)$_2$vitamin D_3 and for sex steroid binding globulin (SSBG), the binding of ketoconazole to human androgen receptors (AR) *in vitro* was also examined. Ketoconazole competition with [^{3}H]methyltrienolone (R1881) for androgen binding sites in dispersed, intact cultured human skin fibroblasts was determined at 22 °C. Fifty percent displacement of [^{3}H]R1881 binding to AR was achieved by 6.4 ± 1.8 (SE) $\times 10^{-5}$ M ketoconazole. Additional binding studies performed with ketoconazole in the presence of increasing amounts of [^{3}H]R1881 showed that the interaction of ketoconazole with AR was competitive when the data were analyzed by the Scatchard method. It should be noted, however, that the dose of ketoconazole required for 50% occupancy of the androgen receptor is not likely to be achieved *in vivo*, at least in plasma. Finally, androgen binding studies performed with other imidazoles, such as clotrimazole, miconazole, and fluconozole, revealed that in this class of compounds only ketoconazole appears to interact with the androgen receptor.

Ketoconazole appears to be the first example of a non-steroidal compound which binds competitively to both SSBG and multiple steroid hormone receptors, suggesting that the ligand binding sites of these proteins share some features in common.

Key words

Ketoconazole – Androgen Receptors – Anti-Androgen – Skin Fibroblasts

Introduction

Ketoconazole is a synthetic imidazole with anti-fungal properties. Since it is effective when given orally, it has become widely used for the treatment of a variety of fungal infections, and has become the treatment of choice for both mucocutaneous candidiasis and North American blastomycosis (*Dismukes, Stamm, Graybill, Craven, Stevens, Stiller, Sarosi, Medoff, Gregg, Gallis, Fields, Marier, Kerkering, Kaplowitz, Cloud, Bowles* and *Shadomy* 1983; *Bradsher, Rice* and *Abernathy* 1985). While therapeutic use of ketoconazole has been infrequently associated with serious side effects (except in patients with previous liver disease), there have been several reports of endocrine dysfunction including, adrenal insufficiency, especially with high dose therapy required in the treatment of life threatening systemic mycoses (*Pont, Williams, Azher, Reitz, Bochra, Smith* and *Stevens* 1982a; *Pont, Williams, Loose, Feldman, Reitz, Bochra, Smith* and *Stevens* 1982b; *Pont, Graybill, Craven, Galgiani, Dismukes, Reitz* and *Stevens* 1984). In men, signs of androgen deficiency, including decreased libido, impotence, gynecomastia, and oligospermia, have been reported in association with the use of ketoconazole. Since decreased testosterone levels have been found in both patients and normal volunteers given ketoconazole, it has generally been assumed that the signs and symptoms of androgen deficiency are secondary to impaired testicular synthesis of testosterone (*Grosso, Boyden, Pamenter, Johnson, Stevens* and *Galgiani* 1983; *Schurmeyer* and *Nieschlag* 1984; *Sikka, Swerdloff* and *Rajfer* 1985; *Kan, Hirst* and *Feldman* 1985).

However, it is possible that the drug may be acting via other means of inducing androgen deficiency besides inhibition of testosterone synthesis such as by increasing androgen clearance, as might occur from alterations of testosterone binding to serum binding proteins, or by competition for androgen binding sites on androgen receptors in target tissues. It is possible, for instance, that binding of ketoconazole to sex steroid binding globulin (SSBG) could play a role in the decreased testosterone levels observed in patients taking ketoconazole chronically. In fact, *Grosso* et al. (1983) have previously shown that ketoconazole displaces steroid hormones from serum transport proteins and, *Glass* (1986) has shown that one week administration of ketoconazole stimulated a rise in serum LH and FSH levels presumably due to the acute lowering of serum testosterone levels.

Interaction of ketoconazole with those proteins responsible for androgen transport and biological activi-

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ty *in vivo* could play a role in the pathogenesis of the hypogonadism observed with therapeutic administration of ketoconazole. In this study, the potential interaction of ketoconazole with human androgen receptors was assessed by using an established *in vitro* assay for measuring the binding of radiolabelled androgens to the androgen binding sites present in cultured human genital skin fibroblasts. The results indicate that ketoconazole possesses affinity for the human androgen receptor protein.

Materials and Methods

Reagents

[17α-methyl-^{3}H] methyltrienolone ([^{3}H]R1881;87 Ci/mmol), nonradioactive methyltrienolone, and [^{3}H] dexamethasone (46 Ci/mmol and Aquasol were obtained from New England Nuclear. Tricine [N-Tris-(hydroxymethyl)methylglycine] and nonradioactive dexamethasone were from Sigma Chemicals. Ketoconazole and miconazole were provided by Janssen Pharmaceutica Inc., Piscataway, NJ. Clotrimazole was provided by Miles Pharmaceuticals, New Brunswick, CT.; fluconazole was provided by Pfizer Inc., Groton, CT. Fetal calf serum (mycoplasma and virus screened), trypsin-EDTA, and penicillin-streptomycin were obtained from Grand Island Biological Co. (Grand Island, NY). Gentamycin was obtained from Schering Pharmaceutical Corp. (Manati, PR). Tissue culture flasks (75 and 150 cm^2) were purchased from Costar (Cambridge, MA), Falcon Plastics (Oxnard, CA), and Corning Glass Works (Corning, NY). Tissue culture medium (IMEMZO) was purchased from Associated Biomedic Systems (Buffalo, NY), and PBS was obtained from Biofluids, Inc. (Rockville, MD).

Cell Culture

Fibroblast cultures were established from genital skin specimens obtained from neonates at routine circumcision. The specimens were finely minced in 60 mm petri dishes containing 4–5 ml improved Eagle's Minimum Essential medium (IMEM) supplemented with 10% fetal calf serum, 10^{-7} M insulin, collagenase (2 mg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and glutamine (0.584 g/liter). This medium was freshly prepared for each sample and filtered through a Swinnex 13 Milipore filter (Millipore Corp., Bedford, MA) immediately before use. After mincing, the mixtures were transferred to 25 cm^2 tissue culture flasks and maintained in the presence of 5% CO_2 in a humidified incubator. After 24 h, the medium was changed to a growth medium consisting of IMEM supplemented with the additives listed above except collagenase. When colonies had grown to approximately 50% confluence (usually within 1–3 weeks), the cells were detached with 0.05% trypsin-0.02% EDTA in PBS at 37 °C and passed serially into larger flasks (75 and 150 cm^2).

Whole Cell [^{3}H]R1881 Binding Assay

The binding of imidazoles to the androgen receptor was determined by competitive displacement of [^{3}H]R1881 from receptor sites in cultured human fibroblasts using a dispersed, intact cell assay system as previously described (*Eil, Lippman* and *Loriaux* 1980; *Eil* and *Edelson* 1984). Briefly, fibroblasts were grown to confluence in five or six 150 cm^2 tissue culture flasks for routine assay. This usually required 4–6 weeks from the time of the initial seeding of the cell line. All studies were performed between passages 3–20. Two days before assay, the medium was changed to one lacking fetal calf serum. This was repeated again 24 hours before assay. The remainder of the procedure was as previously described (*Eil* and *Edelson* 1984), with the binding studies performed at 22 °C to minimize metabolism of the radioligand and competitors, except that [^{3}H]R1881 was substituted for [^{3}H]DHT. Competition assays were performed with 0.5–1.0 nM [^{3}H]R1881 and increasing amounts of the nonradioactive compounds. Binding to low affinity sites was determined in the presence of 5×10^{-7} M R1881 and was subtracted from whole cell binding of

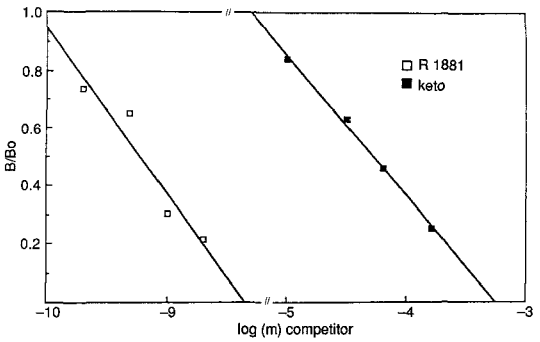

Fig. 1 Ability of ketoconazole (■) and R1881 (□) to compete for [^{3}H]R1881 binding to androgen binding sites in dispersed intact human fibroblasts. $B/B_o = 1$ represents the whole cell binding of [^{3}H]R1881 in the absence of any inhibitor minus low affinity binding (plotted as $B/B_o = 0$) of [^{3}H]R1881 determined in the presence of 5×10^{-7} M R1881. The data points plotted are the mean of triplicate determinations.

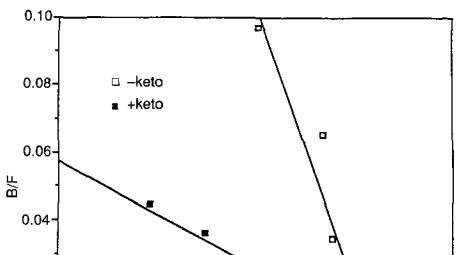

Fig. 2 Scatchard plot of [^{3}H]R1881 binding to dispersed, intact human foreskin fibroblasts performed with increasing concentrations of [^{3}H]R1881 in the absence (□), or presence (■) of 4×10^{-5} M ketoconazole. The data points plotted are the mean of duplicate determinations.

[^{3}H]R1881 obtained in the absence of any inhibitor to assess binding to 5 high affinity sites. All compounds were dissolved in ethanol and added in small aliquots to each tube. The ethanol was allowed to evaporate before addition of the cell suspension and the radioligand, both of which were prepared in IMEM not containing serum or insulin. Although the normal cell line used varied, standard amounts of unlabelled R1881 (0.2, 0.5, 1.0, 2.0 and 500 nM) were included in each assay as an internal control. The amount of R1881 required for 50% displacement of [^{3}H]R1881 was 0.50 ± 0.08 (SE) nM (5 assays), identical to the Kd determined directly with increasing amounts of [^{3}H]R1881 (*Eil* and *Edelson* 1984). The percent binding of [^{3}H]R1881 in the absence of unlabelled hormone was 1.0–5.0%; binding to low affinity sites was 5–10% of the total bound radioactivity. The number of cells added per tube ($0.5–2.0 \times 10^6$) determined these percentages. Individual data points were determined in duplicate. Binding assays with [^{3}H]dexamethasone were performed in an analogous fashion.

Results

Binding of ketoconazole to the androgen receptor was examined by determining the ability of increasing amounts of the compound to displace [^{3}H]R1881 from the androgen receptors in cultured human fibroblasts (Figure 1).

While it is clear that ketoconazole can interact with androgen receptors *in vitro*, a concentration of 6.4 ± 1.8 (SE) $\times 10^{-5}$M (n = 3) ketoconazole was required for 50% displacement of the tracer from the androgen receptor in this system. Similar results were obtained when the binding assay was performed at 37 °C. The relative binding activity of ketoconazole for binding to the androgen receptor at 22 °C, calculated as the ratio of the concentration of unlabelled R1881 to the concentration of ketoconazole required for 50% displacement of the [^{3}H]R1881 was 7.8×10^{-6}. Scatchard analysis of additional binding studies performed with a fixed amount of ketoconazole (4×10^{-5} M) and increasing concentrations of [^{3}H]R1881 indicates that ketoconazole interacts with androgen receptors in a competitive fashion (Figure **2**). No displacement of [^{3}H]R1881 occurred when other imidazole agents used therapeutically – clotrimazole, miconazole, and fluconazole – were tested in this assay system, at doses up to 4×10^{-4} M each (data not shown).

Ketoconazole also competed for [^{3}H]dexamethasone binding to fibroblast glucocorticoid receptors with a displacement curve parallel to unlabelled dexamethasone (results not shown). Fifty percent inhibition of [^{3}H]dexamethasone binding was achieved by 3×10^{-4}M ketoconazole.

Discussion

With the increasing use of ketoconazole as an oral fungicide, additional aspects of its clinical pharmacology are becoming apparent (*Loose, Kan, Hirst, Marcus* and *Feldman* 1983a; *Feldman* 1986). Due to the fact that ketoconazole is an inhibitor or cytochrome p450 enzyme systems (*Sikka, Swerdloff* and *Rajfer* 1985; *Sonino* 1987), it is not surprising that both adrenal and testicular steroidogenesis, each requiring multiple hydroxylation reactions, are inhibited both *in vitro* and *in vivo*. However, the enzyme blockade *in vivo* must only be partial, since patients on long-term therapy with conventional doses of ketoconazole usually do not develop adrenal insufficiency or hypogonadism, suggesting that the pituitary is able to override the glandular dysfunctions by increasing ACTH and LH secretion. Nonetheless, this inhibitory effect of ketoconazole on steroidogenesis has been exploited clinically to treat successfully both ACTH and non-ACTH dependent hypercortisolism and gonadotropin-independent familial testotoxicosis (*Engelhardt, Mann, Hormann, Braun* and *Karl* 1983; *Shepard, Hoffert, Evans, Emery* and *Trachtenberg* 1985; *Sonino, Boscaro, Merola* and *Mantero* 1985; *Contreras, Altieri, Liberman, Gac, Rojas, Ibarra, Ravanal* and *Sèron-Ferrè* 1985; *Holland, Fishman, Bailey* and *Fazekas* 1985).

Based on observations by *Loose, Stover* and *Feldman* (1983b) that ketoconazole binds to rat glucocorticoid receptors and *Hackney* and *Schwartz* (1988) that the drug competed for [^{3}H]dexamethasone binding in a human leukemia cell line, it is also possible that some of ketoconazole's glucocorticoid antagonist activity may be occurring by virtue of occupancy of glucocorticoid receptor sites in target tissues. We confirmed this observation by demonstrating ketoconazole's inhibition of [^{3}H]dexamethasone binding in dispersed human skin fibroblasts. Because certain drugs which induce gynecomastia, such as cimetidine (*Hall* 1976) and spironolactone (*Loriaux, Menard, Taylor, Pita* and *Santen* 1976; *Huffman, Kampmann, Hignite* and *Azarnoff* 1978), are believed to have anti-androgenic properties on the basis of their ability to interfere with androgen binding to its receptor (*Eil* and *Edelson* 1984), ketoconazole was tested for this property as well. The results of the studies reported here indicate that ketoconazole is a competitive inhibitor of the human fibroblast androgen receptor, albeit with relatively weak affinity. Its potency in this regard is similar to that of cimetidine (*Eil* and *Edelson* 1984). While the dose of ketoconazole required for 50% occupancy of the androgen receptor is not likely to be achieved *in vivo* in serum by routine anti-fungal therapy, it is possible that the drug may be concentrated in target tissues, such as the breast, allowing for sufficient levels to interact with the receptor. Finally, individuals with diminished metabolic clearance of ketoconazole might achieve particularly high serum levels of the drug while on standard anti-fungal doses. Since there are no progesterone receptors in fibroblasts (*Eil, Lippman* and *Loriaux* 1980), it is extremely unlikely that ketoconazole is displacing [^{3}H]R1881 from sites other than androgen receptors. Finally, the data presented here do not distinguish between ketoconazole being an androgen receptor antagonist or agonist. This would require additional studies with bioassays.

It is interesting to note that, while these studies were in progress, *Ayub* and *Levell* (1989) reported that ketoconazole along with a number of other imidazole drugs had little or no effect on [^{3}H]R1881 binding to human prostate cytosol androgen receptors. The only possible explanation for the discrepant results between the two studies regarding ketoconazole is that the current assay is performed at 22 °C with intact cells, while the prostate receptor assay is performed at 4 °C with cell extracts. Under our conditions at the higher temperature it is conceivable that ketoconazole is metabolized to a compound with affinity for the androgen receptor. Additional studies at 37 °C confirmed the observations of 22 °C. However, other studies have shown that ketoconazole binds to other receptors and serum binding proteins at 4 °C (*Grosso* et al. 1983; *Loose, Stover* and *Feldman* 1983b; *Hodge, Eil, DeGrange, Liberman, Marx* and *Dunn* 1984; *Hackney* and *Schwartz* 1988; *Ayub* and *Levell* 1989). Furthermore, previous studies with numerous steroids and non-steroidal compounds using the present assay have validated its accuracy and reliability (*Eil* and *Edelson* 1984). In any case, from the studies performed here it appears that ketoconazole's interaction with the androgen receptor is competitive and not artifactual.

The demonstration that ketoconazole interacts with glucocorticoid (*Loose, Stover* and *Feldman* 1983; *Hackney* and *Schwartz* 1988; androgen, and also 1,25-dihydroxyvitamin D (*Hodge* et al. 1984) receptors, as well as SSBG, suggests that the ligand binding sites of these proteins share some features in common. On the other hand, recent work by *Svec* (1988) indicates that ketoconazole may interact with the glucocorticoid receptor at a site other than the agonist binding site. The data shown in Figure **2** indicate that, at least for the androgen receptor, ketoconazole appears to act as a competitive inhibitor, most likely at the ligand binding site. In either case, ketoconazole, or other imidazole derivatives, may prove useful in probing the structure and function of steroid and vitamin D receptor proteins, especially now that the primary amino acid sequences of most of these proteins are known.

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References

Ayub, M., M. J. Levell: The effect of ketoconazole-related imidazole drugs and antiandrogens on [^{3}H]R1881 binding to the prostatic androgen receptor and [^{3}H]5α-dihydrotestosterone and [^{3}H]cortisol binding to plasma proteins. J. Steroid Biochem. 33: 251–255 (1989)

Bradsher, R. W., D. C. Rice, R. S. Abernathy: Ketoconazole therapy for endemic blastomycosis. Ann. Intern. Med. 103: 872–879 (1985)

Contreras, P., E. Altieri, C. Liberman, A. Gac, A. Rojas, A. Ibarra, M. Ravanal, M. Sèron-Ferrè: Adrenal rest tumor of the liver causing Cushing's syndrome: treatment with ketoconazole preceding an apparent surgical cure. J. Clin. Endocrinol. Metab. 60: 21–28 (1985)

Dismukes, W. E., A. M. Stamm, J. R. Graybill, P. C. Craven, D. A. Stevens, R. L. Stiller, G. A. Sarosi, G. Medoff, C. R. Gregg, H. A. Gallis, B. T. Fields Jr., R. L. Marier, T. A. Kerkering, L. G. Kaplowitz, G. Cloud, C. Bowles, S. Shadomy: Treatment of systemic mycoses with ketoconazole: emphasis on toxicity and clinical response in 52 patients. Ann. Intern. Med. 98: 13–20 (1983)

Eil, C., M. E. Lippman, D. L. Loriaux: A dispersed-whole cell method for the determination of androgen receptors in human skin fibroblasts. Steroids 35: 89–104 (1980)

Eil, C., S. K. Edelson: The use of human skin fibroblasts to obtain potency estimates of drug binding to androgen receptors. J. Clin. Endocrinol. Metab. 9: 51–55 (1984)

Engelhardt, D., K. Mann, R. Hormann, S. Braun, H. J. Karl: Ketoconazole inhibits cortisol secretion of an adrenal adenoma *in vivo* and *in vitro*. Klin. Wochenschr. 61: 373–375 (1983)

Feldman, D.: Ketoconazole and other imidazole derivatives as inhibitors of steroidogenesis. Endocr. Rev. 7: 409–420 (1986)

Glass, A. R.: Ketoconazole-induced stimulation of gonadotropin output in men: basis for a potential test of gonadotropin reserve. J. Clin. endocrinol. Metab. 63: 1121–1125 (1986)

Grosso, D. S., T. W. Boyden, R. W. Pamenter, D. G. Johnson, D. A. Stevens, J. N. Galgiani: Ketoconazole inhibition of testicular secretion of testosterone and displacement of steroid hormones from serum transport proteins. Antimicrob. Agents Chemother. 23: 207–212 (1983)

Hackney, J. F., D. A. Schwartz: Ketoconazole inhibition of glucocorticoid action in the human lymphoblastic leukaemia cell line CEM-C7. J. Steroid Biochem. 29: 15–19 (1988)

Hall, W. H.: Breast changes in males on cimetidine. N. Engl. J. Med. 295: 841 (1976)

Hodge, J. W., C. Eil, D. A. DeGrange, U. A. Liberman, S. J. Marx, J. F. Dunn: Ketoconazole: a 1,25-Dihydroxyvitamin D receptor antagonist. Presented at the 6th Annual Meeting of the American Society for Bone and Mineral Research, Hartford, CT, June 1984. (1984, Abstract)

Holland, F. J., L. Fishman, J. D. Bailey, A. T. A. Fazekas: Ketoconazole in the management of precocious puberty not responsive to LHRH-analogue therapy. N. Engl. J. Med. 312: 1023–1028 (1985)

Huffman, D. H., J. P. Kampmann, C. E. Hignite, D. L. Azarnoff: Gynecomastia induced in normal males by spironolactone. Clin. Pharmacol. ther. 24: 465–473 (1978)

Kan, P. B., M. A. Hirst, D. Feldman: Inhibition of steroidogenic cytochrome P-450 enzymes in rat testis by ketoconazole and related imidazole anti-fungal drugs. J. Steroid Biochem. 23: 1023–1029 (1985)

Loose, D. S., P. B. Kan, M. A. Hirst, R. A. Marcus, D. Feldman: Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes. J. Clin. Invest. 71: 1495–1499 (1983a)

Loose, D. S., E. P. Stover, D. Feldman: Ketoconazole binds to glucocorticoid receptors and exhibits glucocorticoid antagonist activity in cultured cells. J. Clin. Invest. 72: 404–410 (1983b)

Loriaux, D. L., A. Menard, A. Taylor, J. C. Pita, R. Santen: Spironolactone and endocrine dysfunction. Ann. Intern. Med. 1976. 85: 630–636 (1976)

Pont, A., P. L. Williams, S. Azhar, R. Reitz, C. Bochra, E. R. Smith, D. A. Stevens: Ketoconazole blocks testosterone synthesis. Arch. Intern. Med. 142: 2137–2140 (1982a)

Pont, A., P. L. Williams, D. S. Loose, D. Feldman, R. E. Reitz, C. Bochra, E. R. Smith, D. A. Stevens: Ketoconazole blocks adrenal steroid synthesis. Ann. Intern. Med. 97: 370–372 (1982b)

Pont, A., J. R. Graybill, P. C. Craven, J. N. Galgiani, W. E. Dismukes, R. E. Reitz, D. A. Stevens: High-dose ketoconazole therapy and adrenal and testicular function in humans. Arch. Intern. Med. 144: 150–153 (1984)

Schurmeyer, T., E. Nieschlag: Effects of ketoconazole and other imidazole fungicides on testosterone biosynthesis. Acta Endocrinol. 105: 275–280 (1984)

Shepard, F. A., B. Hoffert, W. K. Evans, G. Emery, J. Trachtenberg: Ketoconazole use in the treatment of ectopic adrenocorticotropic hormone production and Cushing's syndrome in small cell lung cancer. Arch. Intern. Med. 145: 863–864 (1985)

Sikka, S. C., R. S. Swerdloff, J. Rajfer: In vitro inhibition of testosterone biosynthesis by ketoconazole. Endocrinology 116: 1920–1925 (1985)

Sonino, N., M. Boscaro, G. Merola, F. Mantero: Prolonged treatment of Cushing's disease by ketoconazole. J. Clin. Endocrinol. Metab. 61: 718–722 (1985)

Sonino, N.: The use of ketoconazole as an inhibitor of steroid production. N. Engl. J. Med. 317: 812–818 (1987)

Svec, F.: Differences in the interaction of RU 486 and ketoconazole with the second binding site of the glucocorticoid receptor. Endocrinology 123: 1902–1906 (1988)

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