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4-PREGNENE-3-ONE-20β-CARBOXALDEHYDE: A POTENT INHIBITOR OF 17α-HYDROXYLASE/$C_{17,20}$-LYASE AND OF 5α-REDUCTASE*

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Summary—The pregnene derivative, 4-pregnene-3-one-20β-carboxaldehyde (22-A) was evaluated as an inhibitor of 17α-hydroxylase/$C_{17,20}$-lyase in rat testicular microsomes and of 5α-reductase in human prostatic homogenates. The effect of the compound *in vivo* was studied in adult male rats. The 22-A demonstrated potent and competitive inhibition of 17α-hydroxylase and $C_{17,20}$-lyase with K_i values 8.48 and 0.41 μM, respectively, significantly below the K_m values for these two enzymes (33.75 and 4.55 μM). This compound also showed potent inhibition of 5α-reductase with a K_i value of 15.6 nM (K_m for this enzyme is 50 nM). By comparison, ketoconazole, a currently studied 17α-hydroxylase/$C_{17,20}$-lyase inhibitor for the treatment of prostatic cancer, showed less potent inhibition of 17α-hydroxylase (K_i 39.5 μM) and $C_{17,20}$-lyase (K_i 3.6 μM) and did not inhibit 5α-reductase. Progesterone which has been reported to inhibit the 17α-hydroxylase/$C_{17,20}$-lyase, did not significantly reduce the production of testosterone by rat testes *in vitro* in comparison to controls, while the same concentration of 22-A demonstrated a 42% reduction of testosterone biosynthesis. When the adult male rats were injected s.c. with 22-A at 50 mg/day/kg for a 2 week period, the testosterone concentrations in the rat sera were significantly lower than control values ($P < 0.05$), whereas serum corticosterone levels did not change. These results suggest that 22-A is a selective potent inhibitor for 17α-hydroxylase and $C_{17,20}$-lyase, but is more potent for the $C_{17,20}$-lyase. The compound also inhibits 5α-reductase, and therefore may reduce biosynthesis of testosterone and dihydrotestosterone effectively. Thus, 22-A may be useful in the treatment of problems associated with the androgen excess and prostatic cancer.

INTRODUCTION

The conversion of C_{21} steroids (pregnenolone and progesterone) to the related C_{19} steroids (androgens) is catalyzed by 17α-hydroxylase/$C_{17,20}$-lyase. Effective inhibitors could be useful in investigating the physiological role of this enzyme complex and as potential treatment for problems associated with androgen excess and infertility. An additional utility for such inhibitors may be the treatment of prostatic cancer. Patients respond to hormone ablative therapy but eventually relapse. However, they may respond to subsequent and different types of hormonal therapy. While orchidectomy eliminates testicular androgens, adrenal androgens may continue to stimulate tumor growth. It is presently unclear whether total ablation of all androgens [1] will be more successful than sequential treatment with different agents. In either case, new types of agents could be of benefit in treating this disease. Several approaches are presently being used. These include estrogens, antiandrogens, gonadotropin (LHRH) agonist and enzyme inhibitors. A number of compounds which exhibit 17α-hydroxylase/$C_{17,20}$-lyase have been described [2–8], but most of these inhibitors were nonspecific and had low potency. Ketoconazole, an active imidazole fungicide, which inhibits 17α-hydroxylase/$C_{17,20}$-lyase and other cytochrome *P*450 steroidogenic enzymes, is used currently to inhibit testosterone biosynthesis [9, 10] in the treatment of patients with advanced prostatic cancer [11, 12]. However, this agent is not highly effective. Also, it reduces cortisol production and has some significant side-effects. In order to identify more potent inhibitors of 17α-hydroxylase/$C_{17,20}$-lyase, we have evaluated a number of 20-substituted pregnene derivatives and determined their structure–activity relationships. Since testosterone is converted by 5α-reductase

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to dihydrotestosterone (DHT), the active androgen in the prostate, effective blockade of this enzyme would be expected to be beneficial in the treatment of prostate cancer. As progesterone is an alternate substrate for the 5α-reductase, we have evaluated the pregnene-derivatives as inhibitors of this enzyme also. Thus, inhibition of 17α-hydroxylase/$C_{17,20}$-lyase as well as the 5α-reductase would block all androgen synthesis (androstenedione, testosterone and dihydrotestosterone). We report here the results of studies with the 22-aldehyde pregnene derivative 4-pregnene-3-one-20β-carboxyaldehyde (22-A).

MATERIAL AND METHODS

Chemicals: [4-^{14}C]progesterone (60 mCi/mmol), [1,2,6,7-^{3}H]progesterone (109.3 Ci/mmol), [1,2,6,7-^{3}H]androstenedione (85.4 Ci/mmol), [7-^{3}H]testosterone (23.3 Ci/mmol), and [4-^{14}C]-testosterone (51.4 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). 17α-Hydroxy[1,2,6,7-^{3}H]progesterone (74 Ci/mmol) and [4-^{14}C]androstenedione (59 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). The purity of the radioactive chemicals was checked by TLC or HPLC prior to use and impure chemicals were purified by TLC. The 4-pregnene-3-one-20-carboxaldehyde and other steroid compounds were purchased from Steraloids Inc. (Wilton, NH). Ketoconazole was purchased from Sigma Chemical Co. (St Louis, MO). The *N,N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxyamide (4-MA) was a gift from Dr G. Rasmusson (Merck, Sharp and Dohme Research Labs, Rahway, NJ). The 4-hydroxyandrostenedione (4-OHA) was prepared in our laboratory as previously described [13]. All other chemicals were purchased from either Sigma Chemical Co. or Aldrich Chemical Co. (Milwaukee, WI) and were of analytical grade or HPLC grade. Si250F-PA silica gel TLC plates were from J. T. Baker Inc. (Philipsburg, NJ). Scintillation cocktail 3a70B was purchased from RPI Corp. (Mount Prospect, IL.) Steroids-^{125}I diagnostic kits for radioimmunoassay were purchased from ICN Biochemicals, Inc. (Costa Mesa, CA). A Rackbeta II liquid scintillation counter (LKB) was employed to measure tritium and ^{14}C and an Automatic gamma counter for ^{125}I (LKB Wallac Oy, Finland).

Enzyme preparations

Rat testicular microsomes. Testes were obtained from 220–250 g adult male Sprague–Dawley rats (Charles River, Wilmington, MA), immediately frozen in liquid nitrogen and stored at −70°C. After thawing in an ice bath, the testes were minced and homogenized in 0.25 M sucrose (1:4, w/v), then centrifuged at 12,000 *g* for 30 min and the resulting supernatant centrifuged at 105,000 *g* for 1 h. The supernatant was then decanted and the pellet containing the microsomes was resuspended in 0.1 M sodium phosphate buffer (pH 7.4) (2.5 g wet weight of tissue/2 ml buffer). All the above procedures were performed at 4°C. The microsomal fraction was stored at −70°C. Just before use, microsomes were diluted to a concentration of 1.25 g wet weight of tissue/10 ml phosphate buffer.

Human prostate homogenates. Tissues from patients with benign prostatic hypertrophy (BPH) were obtained fresh from the Pathology Department of the University of Maryland and stored at −70°C until assayed. Tissue was homogenized in 0.1 M phosphate buffer (60 mg wet weight of tissue/ml buffer, pH 7.4) at 4°C and the homogenates were diluted with an equal volume of buffer before use [14].

The protein concentrations of tissue used in each assay were determined by the method of Lowry *et al.* [15].

Enzyme assays

17α-Hydroxylase. The 17α-hydroxylase activity was measured by incubation of the substrate [4-^{14}C]progesterone (various concentration, 10^5 dpm) with 50 μl of an NADPH generating system (NADP 0.65 mM; glucose-6-phosphate 7.1 mM; glucose-6-phosphate dehydrogenase 1.25 IU in phosphate buffer) and approx. 1 mg rat testicular microsomal protein in 450 μl phosphate buffer, pH 7.4, for 30 min under oxygen at 37°C. The incubation was stopped by cooling the mixture in an ice bath and adding 2 ml ether to each incubation tube. The ^{3}H-labeled steroids (progesterone; 17α-hydroxyprogesterone, androstenedione and testosterone, approx. 8000 dpm each) were added as recovery markers and the steroids were extracted with ether (2 ml × 3). The ether was evaporated with air. Steroid carriers (progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone, 5 μg each) were added to the extracts which were then applied to TLC plates for separation of the steroids. After development for 1.5 h in hexane–ethyl acetate (65:35, v/v), the steroids were located by u.v. (254 nm) absorption, scraped off and eluted into

scintillation vials with ether. After evaporating the ether, the steroids were dissolved in counting cocktail and the radioactivity measured. The recovery of steroids calculated from the ^{3}H-labeled markers was between 80 to 90% and the enzyme activity was determined from the percentage conversion of [4-^{14}C]progesterone to 17α-hydroxyprogesterone, androstenedione and testosterone.

$C_{17,20}$-lyase. The procedure for measurement of the $C_{17,20}$-lyase was similar to the procedure for the 17α-hydroxylase, except that the substrate was 17α-hydroxy-[1,2,6,7-^{3}H]progesterone (various concentrations, 10^5 dpm), protein concentration was 0.4 mg and recovery markers were ^{14}C-labeled steroids. Carrier steroids (17α-hydroxyprogesterone, androstenedione and testosterone) were also added to locate the product areas on the TLC plates. The enzyme activity was determined from the percent conversion of 17α-hydroxy-[1,2,6,7-3H]-progesterone to androstenedione and testosterone.

The purity of each steroid after chromatography as described above was confirmed by two methods. (1) The percent conversion of half the extract from an incubation was compared after separation on TLC and HPLC. The HPLC procedure has been described in detail previously [14]. The solvent system used was methanol–acetonitrile–water (15.6:36.4:48); the flow rate was 1 ml/min and pressure 2200 psi. The retention time of these steroids was progesterone 38.3 min, 17α-hydroxyprogesterone 15.93 min, androstenedione 14.33 min, and testosterone 11.43 min. (2) After the first separation by TLC in the solvent system hexane–ethyl acetate (65:35), the eluted steroids were chromatographed in a second solvent system chloroform–acetonitrile (85:15).

5α-Reductase. [7-^{3}H]testosterone (0.1–10 μM, 10^6 dpm) was incubated for 30 min at 37°C with the NADPH generating system, homogenates of human prostatic tissue (30 mg wet weight of tissue/1 ml buffer) and the candidate inhibitors at concentrations ranging from 0.1–10 μM. Steroids ^{14}C-labeled (testosterone, androstenedione and DHT) and authentic markers [testosterone, androstenedione, DHT, 5α-androstane-3α-diol and -3β-diol, (3α and 3β-diols)] were added after the incubations. The steroids were extracted with ether and the extracts chromatographed on TLC (chloroform–ether, 95:5). The DHT and the diols were located from their markers after exposure of the plate to iodine vapor. The steroids were scraped from the plate and radioactivity measured. Results were calculated from the percentage conversion of [7-^{3}H]-testosterone to DHT, the 3α- and 3β-diols.

In order to confirm the purity of the metabolites, portions of several samples were chromatographed on TLC in a second solvent system (ether–hexane, 1:2).

Determination of K_m and K_i. The kinetic parameters for the 17α-hydroxylase, the $C_{17,20}$-lyase and the 5α-reductase were obtained by Lineweaver–Burk plots at various substrate and inhibitor concentrations.

In vivo studies

A group of 7 adult male Sprague–Dawley rats were injected s.c. with vehicle (0.3% hydroxypropyl cellulose, HPC) and a group of 8 rats were injected s.c. daily with a suspension of 22-A in HPC (50 mg/kg/day) for 2 weeks. Blood samples (about 1 ml per rat) were collected prior to the first injection and then 2–3 h before each injection on day 1, 3, 6, 9, and 13 from the carotid vein. The sera were separated by centrifugation at 2200 rpm and stored at −15°C until assayed. Testosterone levels in the rat sera were measured by radioimmunoassay using the [^{125}I]testosterone diagnostic kits. The procedure was modified by the addition of steroid-free rat sera (prepared by treating the sera with charcoal) to each tube of the standard curve and an equal amount of the testosterone-free human sera (provided with the kit) to the sample tubes [16]. The corticosterone levels in the rat sera were measured using the [^{125}I]corticosterone kits without modification. The results obtained were analyzed by Analysis of Variance followed by the Duncan's multiple range test.

RESULTS

In rat testicular microsomes, the K_m value determined for the 17α-hydroxylase was 33.85 μM and for the lyase was 4.55 μM; V_{max} values were 420 and 180 pmol/min/mg protein, respectively.

The compound 22-A showed competitive inhibition for both the hydroxylase and the lyase, with a K_i value of 8.48 and of 0.41 μM, respectively, significantly below their K_m values (Figs 1 and 2 and Table 1). Thus, 22-A is a better inhibitor of the lyase than of the hydroxylase. In comparison, the K_i values of 17α-hydroxylase and $C_{17,20}$-lyase for ketoconazole were 39.5 and 3.6 μM, respectively, which are similar to their

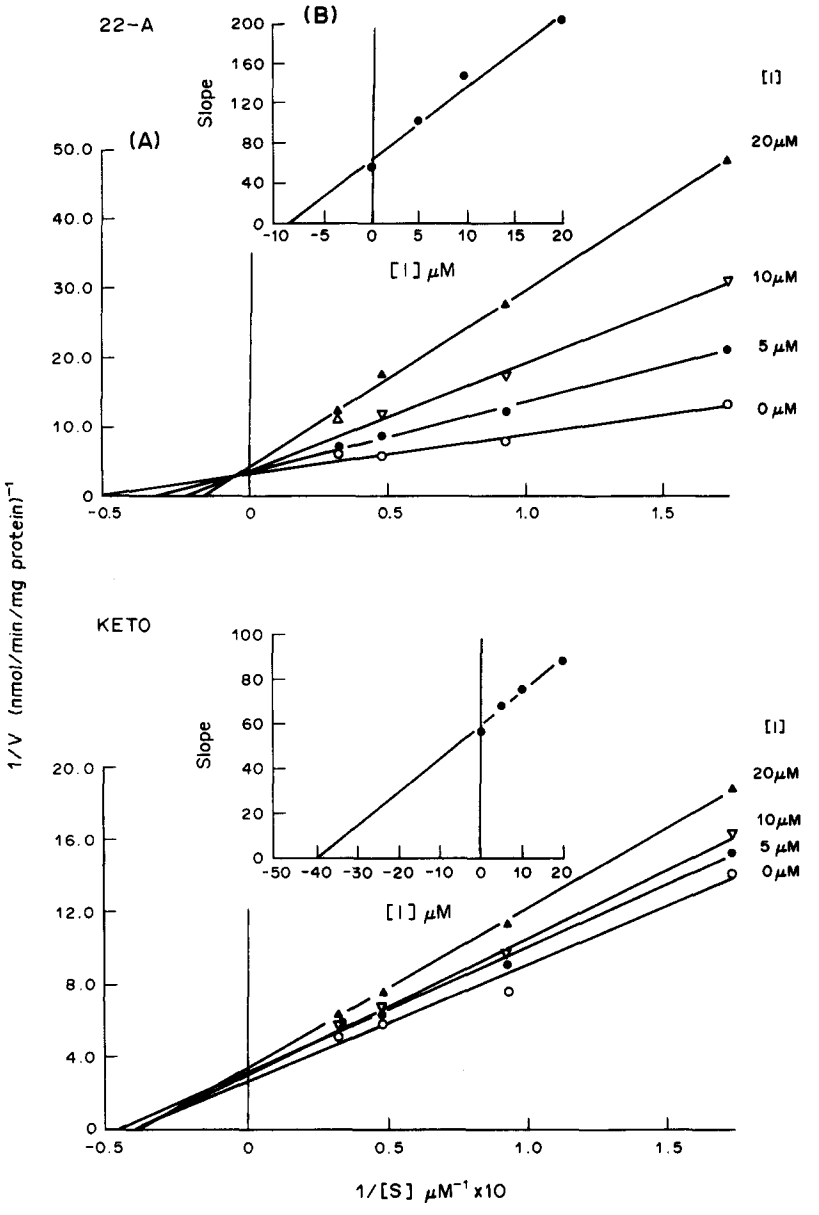

Fig. 1. Inhibition of 17α-hydroxylase by 22-A (above) and ketoconazole (below). (A) Lineweaver–Burk plots of enzyme activities at varying substrate and inhibitor concentrations; (B) replot of the slope of each reciprocal plot vs [I]. The 22-A showed competitive inhibition with a K_i value of 8.5 μM and ketoconazole showed mixed-type inhibition with a K_i value of 39.5 μM.

K_m values. We also tested 4-MA, known to be a 5α-reductase inhibitor [22] and 4-OHA, an aromatase inhibitor [13], but these compounds did not inhibit the 17α hydroxylase/$C_{17,20}$-lyase enzyme.

As progesterone is reported to inhibit this enzyme [17], we investigated its effect on conversion of radiolabeled substrate to androgens. While the percentage converted was inhibited by the addition of 20 μM progesterone, the production of androstenedione and testosterone (nmol) was only reduced by 38.5%. This decline was mainly due to a reduction in androstenedione production, whereas there was no significant decrease in the amount of testosterone produced (Tables 2 and 3). In contrast to progesterone, the same concentration of 22-A caused 68% inhibition of the $C_{17,20}$-lyase (Table 2) and 42% reduction in testosterone concentrations (Table 3). Unlike progesterone, there was no conversion of 22-A to testosterone (Table 3).

The above compounds were also evaluated as inhibitors of 5α-reductase in human prostatic

tissue. Due to the availability of tissue from patients with BPH, rather than from those with prostatic cancer or normal subjects, we have used this tissue as a source of prostatic 5α-reductase. The K_m for the human prostatic 5α-reductase was 50 nM and the V_{max} was 2.24 pmol/min/mg protein. Compound 22-A caused a reduction in the conversion of testosterone into DHT and the 3α- and 3β-diols; the K_i value was determined to be 15.6 nM (Table 1). In comparison, the K_i value of 4-MA for the 5α-reductase was 8.4 nM.

Studies of the effect of 22-A treatment on serum steroid levels were carried out in mature male rats. Serum testosterone levels were significantly lower than control levels by day 3 of treatment and remained low throughout the 2 week treatment ($P < 0.05$) as indicated in Fig. 3. No effect of treatment on serum corticosterone concentrations was observed (data not shown).

DISCUSSION

The 17α-hydroxylation and removal of the $C_{17,20}$ side-chain have been reported as two reactions catalyzed by a single protein [18, 19]. We observed that the K_m values for these two enzymatic reactions were significantly different.

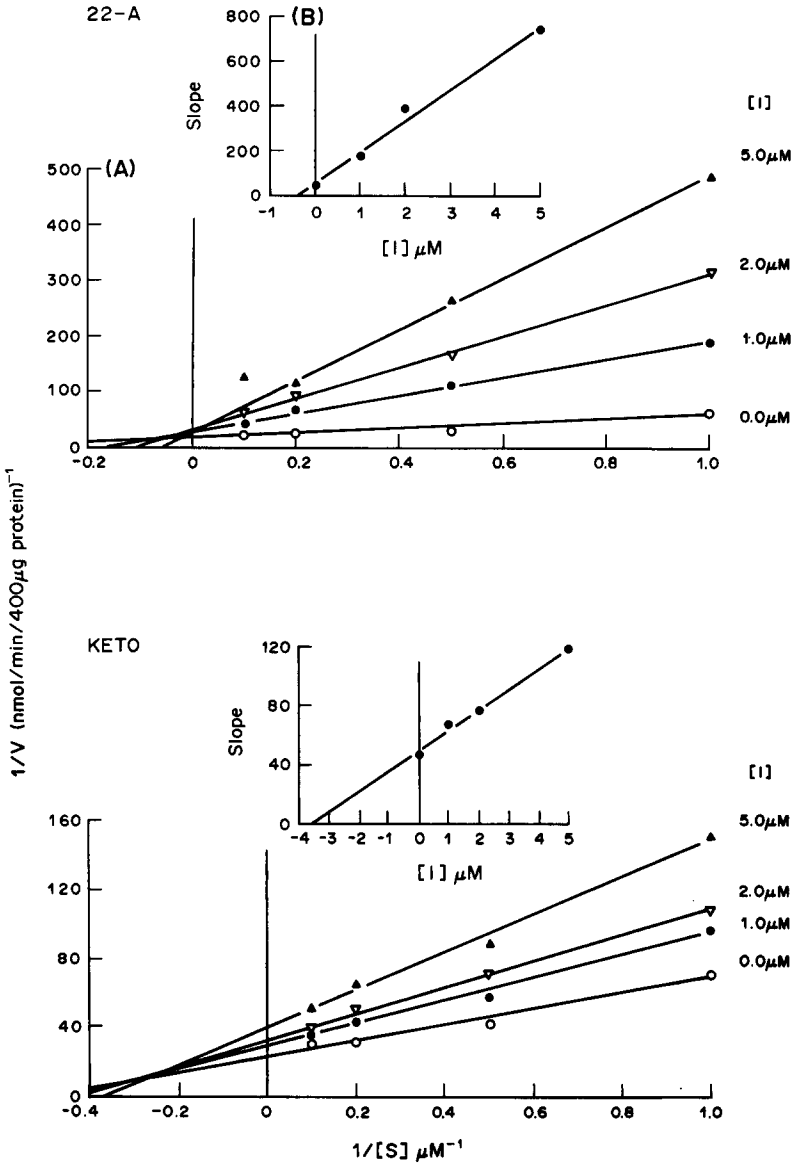

Fig. 2. Inhibition of $C_{17,20}$-lyase by 22-A (above) and ketoconazole (below). (A) and (B) refer to same plot methods as in Fig. 1. The 22-A showed competitive inhibition with a K_i value of 0.41 μM, significantly below the K_m value of the enzyme. In comparison, the K_i value for ketoconazole was 3.6 μM with a mixed-type of inhibition rather than competitive inhibition as previously reported [5].

Table 1. Inhibition of rat 17α-hydroxylase and $C_{17,20}$-lyase[a] and human 5α-reductase[b]

Compound	K_i (μM) 17α-Hydroxylase	$C_{17,20}$-lyase	K_i (nM) 5α-reductase
22-A	8.48	0.41	15.6
Keto	39.50	3.60	NI
4-MA	NI[c]	NI	8.4

[a]Rat testicular microsomes were incubated with [4-^{14}C]progesterone or 17α-hydroxy[1,2,6,7-^{3}H]progesterone (various concentrations, 10^5 dpm), various concentrations of inhibitors, and NADPH generating system at 37°C under O_2 for 30 min.
[b]Homogenates of prostatic tissue were incubated with [7-^{3}H]testosterone (various concentrations 7×10^5 dpm) and various concentrations of inhibitors, under conditions as above.
[c]NI is no inhibition.

Table 3. Testosterone concentrations in incubations of rat testicular microsomes[a]

Compound (10 nmol)	Substrate	Testosterone (nmol)
None	P4	1.15
P_4	P4	1.21
22-A	P4	0.67
None	0	0.0021
22-A	0	0.0028

[a]Incubation was performed with or without progesterone (P_4) substrate (10 nmol). No radiolabeled substrate was added. Other conditions were the same as those for Table 2. Testosterone concentrations were measured by radioimmunoassay.

The K_m value for the hydroxylase was much greater than for the lyase, which is consistent with high concentrations of endogenous progesterone. Our results showed that 22-A preferentially inhibited the lyase rather than the hydroxylase step. Compound 22-A is approx. 9-fold more potent than ketoconazole for the rat testicular $C_{17,20}$-lyase according to the K_i values. We also found that ketoconazole exhibits a mixed-type of inhibition of this enzyme (Figs 1 and 2) rather than a competitive inhibition as previously reported [5].

In earlier studies, it had been noted that progesterone inhibits 17α-hydroxylase/$C_{17,20}$-lyase [17]. Therefore, a comparison between 22-A and progesterone for inhibition of 17α-hydroxylase/$C_{17,20}$-lyase was carried out. Although the $C_{17,20}$-lyase was partially inhibited by the high concentration of progesterone, the overall amount of testosterone produced (from the additional amount of progesterone and the substrate concentration which totaled 40 μM progesterone), was not significantly different from the control value. In contrast to progesterone, 22-A was not converted to testosterone and there was a significant reduction in the concentration of testosterone produced (Tables 2 and 3).

Table 2. The effect of 22-A and progesterone on the inhibition of 17α-hydroxylase/$C_{17,20}$-lyase

Compound (10 nmol)	Products (nmol) 17OHP$_4$	A	T	Inhibition (%) 17α-OHase	$C_{17,20}$-lyase
None	2.64	1.60	0.84	0	0
P_4	2.94	0.78	0.72	12.6	38.5
22-A	0.88	0.44	0.34	67.3	68.0

Incubations were performed with [7-^{3}H]progesterone (20 μM, 10^5 dpm), 22-A or additional progesterone (P_4), rat testicular microsomes (approx. 1 mg protein) and NADPH generating system in sodium phosphate buffer (pH 7.4, total volume 0.5 ml) at 37°C under oxygen for 30 min. The 17α-hydroxylase (17α-OHase) products were calculated from the percent conversion of progesterone to 17α-hydroxyprogesterone (17OHP$_4$), androstenedione (A) and testosterone (T); The $C_{17,20}$-lyase products were calculated from the overall conversion of progesterone to androstenedione and testosterone.

No adverse effects of treatment were apparent in the rats administered 22-A. There was considerable variation in serum testosterone levels from day to day in untreated rats. Values on day 0 and 1 were unusually low, being significantly lower than those on all other days. The compound maintained a significant reduction in testosterone values from day 3 throughout the treatment period, suggesting that this compound is active *in vivo*. While serum testosterone levels were reduced to about the same extent as occurs with ketoconazole treatment at a higher dose (75 mg/kg/day) [20], they were not as low as in castrated rats [21]. Further studies are in progress to determine the effect of 22-A on intraprostatic concentrations of DHT and on the weight of the prostate. The compound did not have any effect on serum corticosterone levels in the rats. In contrast to similar studies with ketoconazole [20], our results suggest that 21-hydroxylase is not inhibited by 22-A. Although we have not yet evaluated the effects of the compound on adrenal steroid biosynthesis in human tissue, the finding that 22-A inhibits the lyase to a greater extent than the 17α-hydroxylase in the rat, suggesting that it may have a sparing effect on cortisol production.

The 5α-reductase inhibitors, 4-MA and finasteride were noted to increase testosterone levels [22, 23]. Although this effect may be beneficial in treating men with BPH without causing gynecomastia and impotence, inhibition of all androgen production is an important goal of treatment for prostatic cancer [1]. The 22-A may be of value in this regard because of its dual action in reducing the production of substrate for, and the activity of the 5α-reductase.

Estrogen receptors have been identified in the prostate and greater than normal levels of estrogen have been detected in the stroma of BPH, which suggested that estrogen may also have a role in this disease. Although our previous

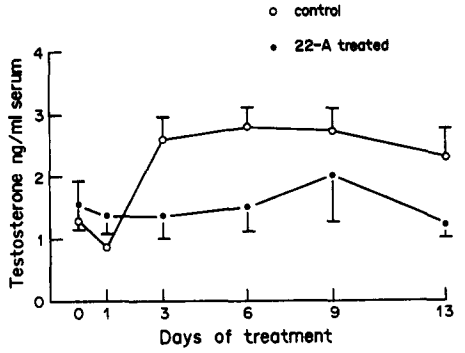

Fig. 3. Serum testosterone levels of rats (mean ± SEM) treated with 22-A (50 mg/kg/day s.c.). Testosterone concentrations were significantly different from those of control rats on day 3 and on the following days measured throughout the 2 week treatment ($P < 0.05$).

studies suggested that the prostate does not possess significant aromatase activity, estrogen produced by peripheral conversion could influence the prostate. In studies of prostatic cancer patients treated with aminoglutethimide, significant subjective responses (60%) were observed. These results were attributed to inhibition of estrogen production since there was no reduction in androgen levels [24, 25]. Similar results have been reported in patients treated with 4-OHA (80% subjective responses) [26]. If estrogen has a role in stimulating prostatic cancers, an additional effect of inhibiting the 17α-hydroxylase/$C_{17,20}$-lyase would be reduction in the estrogen production.

In conclusion, our results suggest that the C-20 substituted pregnene derivative, 22-A is an effective inhibitor of the 17α-hydroxylase/$C_{17,20}$-lyase, with greater potency for the $C_{17,20}$-lyase. The compound is a more potent inhibitor than ketoconazole. In addition, 22-A effectively inhibits 5α-reductase in human prostatic tissue. The 22-A also appears to be active *in vivo* as indicated by a reduction in serum testosterone levels in rats treated with the compound. Although 22-A inhibits both the 17α-hydroxylase/$C_{17,20}$-lyase and 5α-reductase, it appears to be selective for these enzymes, as it had no effect on corticosterone biosynthesis in the rat. Further studies of this and other pregnene inhibitors are in progress.

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