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EVALUATION OF SOME 4-FLUORO- AND 4-CYANO DERIVATIVES OF Δ^4, 3-KETOSTEROIDS AS INHIBITORS OF TESTOSTERONE 5α-REDUCTASE

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Some 4-fluorinated analogues of 3-oxo-Δ^4 steroids and 4-cyano derivatives of progesterone and androstenedione were evaluated as inhibitors of steroid 5α-reductase activity. Inhibitors of this enzyme may be useful in treating prostatic cancer. 4-Fluoroandrostenedione was a modest inhibitor of the rat enzyme (IC_{50} = 4.08 μM), while 4-cyanoprogesterone was a potent inhibitor of both the rat and human enzymes (IC_{50} values = 0.045 μM and 0.050 μM respectively). These two steroids were tested *in vivo* for activity against androgen sensitive organs in WHT mice. 4-Fluoroandrostenedione caused increases in organ weights, suggesting it is an androgen agonist, while the 4-cyano compound displayed modest androgen ablation. Therefore substitutions at the 4-position may produce compounds of therapeutic use in treating prostate cancer.

KEY WORDS: 4-Fluoroandrostenedione, 4-cyanoprogesterone, Δ^4, 3-ketosteroids, steroid 5α-reductase

INTRODUCTION

The principal active androgen within the prostate is 5α-dihydrotestosterone (DHT). Testosterone, the major plasma androgen, is reduced to DHT by the enzyme, steroid 5α-reductase (NADPH : Δ^4-3-oxosteroid-5α-oxidoreductase) which is located within the prostate gland. Recent data has demonstrated the existence of two isozymes of 5α-reductase (designated type 1 and type 2), encoded by different genes.[1,2] The physiological role that these two isozymes play in androgen action remains to be established. Nevertheless, inhibition of 5α-reductase activity is an attractive target for the treatment of androgen- dependent prostatic disease, since circulating levels of testosterone, and hence libido, should be preserved. Very recently one such inhibitor, the 4-azasteroid, finasteride, which has a K_i value in the nM range, has been clin-

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ically evaluated as a treatment both for benign prostatic hyperplasia[3] and prostatic carcinoma.[4] Among other types of steroidal structures which have shown promising inhibitory activity towards this target enzyme are 3-oxo-Δ^4-steroids having electron-withdrawing substituents at the 4-position. Thus both 4-chlorotestosterone and 4-chloro-17α-hydroxyprogesterone inhibited 5α-reductase activity in rat and human prostate in organ culture, causing 50% inhibition at 2.5 μM.[5] More potent inhibitors, comparable to the 4-azasteroids are 4-cyano-substituted 3-oxo-Δ^4-steroids having unnatural 17β-substituents in particular the 17β-diethylaminocarbonyl derivative with an IC_{50} against the rat enzyme[6] of 2.9×10^{-8}M. These literature precedents prompted the present studies in which we have evaluated 4-fluorinated analogues of some 3-oxo-Δ^4-steroids and 4-cyano-derivatives of the natural steroids androstenedione and progesterone as inhibitors of steroid 5α-reductase activity.

MATERIALS AND METHODS

Chemical Syntheses

The synthesis of the following compounds has been described: 4-fluoroandrost-4-ene-3,17-dione, 4-fluorotestosterone and 4-fluoro-19-norandrost-4-ene-3,17-dione[7]; 4-fluoroprogesterone[8]; 4-fluoro-19-nortestosterone[9] and 4-cyanoprogesterone.[10] The synthesis of 4-cyanoandrostenedione follows the precedent of a literature route to 4-cyanosteroid,[6] in which addition of cyanide to steroidal 4,5-epoxides produced 4,5-cyano products which were then pyrolysed to yield the desired 4-cyano steroids.

4-Cyanoandrost-4-en-3,17-dione. A solution of sodium cyanide (7.4 g, 0.15 mole) in water (20 ml) was added to 4,5-epoxyandrostan-3,17-dione (ca 4:1 ratio β:α, 5.0 g, 0.16 mole) in ethanol (250 ml). The resultant solution was heated under reflux for 22 h, then cooled to room temperature and concentrated *in vacuo*. A solution in water (100 ml) of the residue was adjusted to pH 4 with 6 M HCl and the products extracted with dichloromethane (4×50 ml), the extracts washed with water (2×50 ml) and brine (50 ml) and concentrated to give yellow crystalline 4,5-dicyanoandrost-4-en-3-ol. The dicyanosteroid (1.0 g, 3 mmol) was heated under reduced pressure (10 Torr) at 235°C until the solid melted and subsequent gas evolution ceased (approx. 12 min). Chromatography of the residue on a column of silica gel, with acetone: ether (1:4) as eluent, gave the title compound as colourless crystals (0.8 g, 87%) from acetone: ether 1:1), m.p. 207–209°C; (Found: C, 76.30; H, 7.75; N, 4.15. $C_{20}H_{25}NO_2$ requires C, 77.12; H, 8.10; N, 4.50%). V_{max} 2240 (C≡N), 1780, 1700 (C=O), 1595 cm^{-1}. Mass spectrum (EI), *m/z* 311 M^+, 100%), 269 ($[M\text{-}CH_2CO]^+$, 59%).

Enzyme Preparation and Assay Procedures

The preparation of steroid 5α-reductase from the ventral prostates of adult Wistar rats and the procedures for assaying inhibitors against the rat enzyme were as previously described.[12] The enzyme activity was determined radiometrically by measuring the conversion of 4-[^{14}C] testosterone into 4-[^{14}C]DHT and separation of prod-

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uct from substrate by thin-layer chromatography. The substrate concentration was 5 μM[^{14}C]testosterone (K_m = 1.4 μM) at an optimum pH of 7.4. All assays were performed under conditions of linearity with respect to time and protein concentration. For the human enzyme assay, benign prostatic tissue was obtained from patients undergoing radical retropubic prostatectomy. The procedure was essentially as described above except the substrate concentration was 2.5 μM (K_m = 0.50 μM) and the optimum pH was 5.4 using 0.1 M succinic acid-NaOH as buffer.[13]

Animal Experiments

Adult male WHT mice (12 weeks old) were treated daily for 14 days after which they were sacrificed and the organs of interest (adrenals, ventral prostate, seminal vesicles and testes) were dissected out and weighed. 4-Fluoroandrostenedione was prepared in steroid suspending vehicle (0.3% hydroxypropylcellulose) and was given subcutaneously. 4-Cyanoprogesterone was prepared in 5% benzyl alcohol, 95% safflower oil and was given intraperitoneally. Vehicle controls received the vehicle only (5 ml/kg). Castration was carried out via the scrotal route while the mice were under pentobarbital anaesthesia. The sham operated group underwent the same surgical procedures as the castrates except that the testes were not removed. After 14 days the animals were sacrificed and the organs removed and weighed. For the results, statistical significance was calculated using one way analysis of variance and the Fishers least significant difference method for multiple comparisons between vehicle control and other groups.

RESULTS

Using the *in vitro* rat assay system, substitution with fluorine at the C-4 position of progesterone does not lead to an increase in potency towards 5α-reductase (Table 1), however with the androstenedione derivatives a 4–5 fold increase in potency occurs, although the IC_{50} values are in the μM range. The 4-cyano steroids (Table 2) are potent inhibitors against both the rat and human 5α-reductase. From these results, 4-fluoroandrostenedione, and 4-cyanoprogesterone were studied *in vivo* to determine if they had any effect on the weight of androgen-dependent organs in WHT mice. Contrasting results were obtained, 4-fluoroandrostenedione, at all the tested doses, increased the weight of the seminal vesicles. At the top dose used, the weight of seminal vesicles was increased by 60%, while prostate weight increased by 29%, whereas testes decreased by 12% (Table 3). On the other hand, 4-cyanoprogesterone caused a decrease in the weight of seminal vesicles by 41% at the top dose and an increase in testicular weight by 11%, though with no dose related trend in prostate weight (Table 4). In comparison, castration produces profound decreases in prostate and seminal vesicle weights (72 and 92% respectively) as shown in Table 5.

DISCUSSION

We have shown that 4-fluoro-substituted steroids have inhibitory activity towards rat prostatic 5α-reductase, in addition to their previously reported activity as inhibitors

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TABLE 1
Effect of Androstenedione, Progesterone and Fluorinated Steroids on Rat Prostatic 5α-Reductase Activity

Steroid	IC_{50} Value (μM)
Androstenedione	20.00±1.40
Progesterone	3.06±0.42
4-Fluoroandrostenedione	4.08±0.38
4-Fluoroprogesterone	3.25±0.78
4-Fluoroandrostenedione	8.50±0.71
4-Fluorotestosterone	3.65±0.21
4-Fluoronortestosterone	3.00±0.10

Data shown are the means of triplicate determinations ± standard error

TABLE 2
Effect of Progesterone and Cyanosteroids on Rat and Human Prostatic 5α-Reductase Activity

Steroid	IC_{50} Value (μM)	
	Rat	Human
Progesterone	3.06±0.42	3.0±0.31
4-Cyanoandrostenedione	0.78±0.08	0.15±0.02
4-Cyanoprogesterone	0.045±0.009	0.05±0.008

Data shown are the means of triplicate determinations ± standard error

TABLE 3
Effect of 4-Fluoroandrostenedione on Weights of Androgen-Sensitive Organs in WHT Mice

Dose (mg/kg)	Mean Weight (mg)			
	Adrenals	Prostate	Seminal Vesicles	Testes
Controls	3.8±0.1	8.8±0.4	223±5	143±5
Vehicle Controls	4.1±0.1	7.8±0.5	173±8	142±3
50	4.4±0.1	7.2±0.5	227±7*	134±6
200	4.5±0.1	8.9±0.7	287±4*	126±4*
1000	5.8±0.3*	10.1±0.7*	278±7*	125±3*

*p = <0.01
Data shown are the means of 5 determinations ± standard error (SE) for drug treated groups and of 10 determinations ± SE for control groups.

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TABLE 4
Effect of 4-Cyanoprogesterone on Weights of Androgen-Sensitive Organs in WHT Mice

Dose (mmol/mg/kg)	Adrenals	Mean Weight (mg) Prostate	Seminal Vesicles	Testes
Controls	3.7±0.4	11.9±1.5	223±5	136±6
Vehicle Controls	5.1±0.1	13.1±1.2	214±10	146±3
0.02	4.8±0.4	10.2±0.6	171±18+	137±2
0.1	4.2±0.3	10.7±1.3	195±20	144±8
0.5	5.0±0.2	10.8±0.9	148±7*	163±6*

*p = < 0.01;+p = < 0.05
Data shown are the means of 5 determinations ± standard error

TABLE 5
Effeet of Castration on Weights of Androgen-Sensitive Organs in WHT Mice

Treatment	Adrenals	Mean Weight (mg) Prostate	Seminal Vesicles	Testes
Controls	4.3±0.1	9.1±0.9	201±6	139±4
Shams	3.9±0.1	9.5±0.5	168±6	126±6
Castrates	3.9±0.1	2.5±0.2*	16±1*	–

*p=< 0.01
Data shown are the means of 5 determinations ± standard error

of human placental aromatase.[7] 4-Fluorotestosterone and 4-chlorotestosterone have similar potencies, showing that this replacement is broadly neutral in its effect on inhibition. As might be expected from the published precedent,[6] 4-cyanoandrostenedione and 4-cyanoprogesterone were much more potent inhibitors, the latter being comparable to the potent azasteroidal inhibitors from which a candidate drug had been selected. Because of their potency against the rat enzyme, these cyano-derivatives were also tested against the human 5α-reductase. 4-Cyanoprogesterone retained its activity against the human enzyme, as did its natural counterpart progesterone, whereas 4-cyanoandrostenedione showed improved activity against the human enzyme.

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The 5α-reductase inhibitory activity conferred by these two electron-withdrawing substituents is in part in contrast to their effect on aromatase inhibitory activity. Thus, the 4-fluoro-derivatives of the C_{19} steroids were highly potent aromatase inhibitors with IC_{50} values of 0.08 μM for 4-fluoroandrostene-dione and 0.2 μM for 4-fluorotestosterone.[7] In contrast, the two 4-cyano-derivatives were weak inhibitors of human placental aromatase with IC_{50} values greater than 5 μM (results not shown).

Two of the compounds, 4-fluoroandrostenedione and 4-cyanoprogesterone, were tested for their effects on androgen-dependent organs *in vivo*. Tests on organ weights in the rat are an established method for evaluating compounds with potential activity against androgen-dependent disease.[14] However because of the quantity of compound required for tests in rats, we have preferred to use WHT mice, having found that androgen sensitive organs (prostate, adrenals, seminal vesicles) could be reproducibly dissected and weighed and that the animals responded appropriately to castration. From the results it would appear that 4-fluoroandrostenedione is an androgen *agonist* in the WHT mouse, possibly through the action of the compound or its metabolites on the androgen receptor. 4-Cyanoprogesterone in contrast shows effects of androgen ablation, though with modest potency compared to castration. One possible cause of the modest potency could be degradation of 4-cyanoprogesterone by the steroidal 17α-hydroxylase/C_{17-20}lyase enzyme in the testes and adrenals to give the less potent inhibitor 4-cyanoandrostenedione, by analogy with the natural pathway whereby progesterone is converted to androstenedione.[15] However, 4-cyanoprogesterone proved to be neither a substrate for this enzyme nor a significant inhibitor (IC_{50} ca. 100 μM) when tested by a published procedure[16] (results not shown).

In conclusion, the present studies have identified 4-cyanoprogesterone as a potent inhibitor of steroidal 5α-reductase, having modest *in vivo* potency in ablating androgen-dependent organs in WHT mice, encouraging the exploration of a range of alternative substituents at the 4-position as one strategy in the development of novel 5α-reductase inhibitors to treat prostatic cancer.

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