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EFFECTS OF 4-HYDROXYANDROST-4-ENE-3,17-DIONE AND ITS METABOLITES ON 5α-REDUCTASE ACTIVITY AND THE ANDROGEN RECEPTOR

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The steroidal aromatase inhibitor, 4-hydroxyandrost-4-ene-3,17-dione (4OHA) and its metabolites, 4-hydroxytestosterone (4OHT), 3β,17-dihydroxy-5α-androstan-4-one (metabolite A) and 3α,17-dihydroxy-5β-androstan-4-one (metabolite B) were evaluated as inhibitors of the human prostatic 5α-reductase enzyme and for binding to the rat prostatic androgen receptor. 4OHA and 4OHT were weak inhibitors of 5α-reductase with IC_{50} values of 15–29 μM. Metabolites A and B had no significant inhibitory activity. 4OHA and metabolites A and B bound weakly to the androgen receptor. The binding affinities (RBA) relative to mibolerone (RBA = 100) were 0.085, 0.485 and 0.016, respectively. However, 4OHT (RBA = 75) was a more potent binder than the endogenous androgen 5α-dihydrotestosterone (RBA = 66). The ability of these metabolites, in particular 4OHT, to bind to the androgen receptor may explain the *in vivo* androgenic activity of 4OHA.

KEY WORDS: 4-Hydroxyandrost-4-ene-3,17-dione, 5α-reductase, androgen receptor.

INTRODUCTION

The first-line therapy of advanced prostatic cancer is androgen deprivation. Such treatment produces responses in 80% of patients, but relapse usually occurs within 18 months. Therapeutic options after relapse are limited. However, blockade of adrenal androgens using aminoglutethimide (AG) with co-administration of replacement

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corticosteroids was found to be of some benefit in relapsed patients.[1-3] The endocrine changes associated with this treatment demonstrated that the clinical effect of AG was not due to androgen suppression.[4,5] AG is an aromatase inhibitor that suppresses estrogens *in vivo* and it has been suggested that this could be its mode of action in advanced prostate cancer.[6]

The more potent and selective aromatase inhibitor, 4-hydroxyandrost-4-ene-3,17-dione (4OHA), has been shown to suppress estrogen levels in female patients and is in clinical trial for the treatment of postmenopausal women with advanced breast cancer.[7-9] Therefore 4OHA was evaluated in advanced prostate cancer patients who had relapsed following castration. There was a subjective response in 63% of patients, and serum estradiol was reduced in most cases. However 40% of patients experienced a tumour flare. There was no correlation between response or tumour flare and changes in steroid levels.[10-12] Furthermore there were differences in the urinary metabolites of 4OHA in prostate cancer patients compared to breast cancer patients. Although no free steroids were detected in either case, glucuronide conjugates of 4OHA and metabolites A and B were found in both the male and female samples, but 4OHT glucuronide was only detected in female urine.[13,14] The mechanism of action of 4OHA in the palliation of prostate cancer and in the induction of tumour flare remains obscure. It has been reported that 4OHA inhibits human prostatic 5α-reductase, the enzyme responsible for converting testosterone into its active metabolite, 5α-dihydrostestosterone[15-17] (DHT) and therefore may modify the intraprostatic metabolism of testosterone. In order to shed light on the activity of 4OHA in prostatic cancer and the mechanism of the tumour flare, we have evaluated 4OHA and several of its metabolites, namely 4-hydroxytestosterone (4OHT), 3β,17-dihydroxy-5α-androstan-4-one (metabolite A) and 3α,17-dihydroxy-5β-androstan-4-one (metabolite B) against the human 5α-reductase enzyme and the rat prostatic androgen receptor.

MATERIALS AND METHODS

Chemicals

[4-^{14}C]-Testosterone (57 mCi/mmol), [17α methyl-^{3}H]-mibolerone (85 Ci/mmol) and radioinert mibolerone were purchased from Amersham International plc (Amersham, Bucks). Triamcinolone acetonide, dithiothreitol (DTT), bovine serum albumin (BSA) and all unlabelled steroids were obtained from Sigma Chemical Co. Ltd, Poole. Dextran T-70 was purchased from Pharmacia Ltd (Milton Keynes, Bucks). Charcoal (Norit "OL") was obtained from Hopkin and Williams (Chadwell Heath, Essex). Thin-layer chromatography was performed on Merck Kieselgel 60 F254 precoated silica gel plates (20 × 20 cm) purchased from BDH Chemicals Ltd. (Poole UK). 4OHA was provided by Ciba-Geigy Pharmaceuticals (Horsham, UK). The metabolites of 4OHA were synthesised as previously described.[13,18,19] All other reagents were of analytical grade. [^{3}H]-Mibolerone was purified prior to use by normal phase silica TLC using chloroform : methanol (19 : 1) as solvent system.

Preparation of Rat Prostate Cytosol and the Androgen Receptor Binding Assay

Ten male Wistar rats (approx. 16 weeks old) were castrated then sacrificed 24 h later, by anaesthesia followed by cervical dislocation. The prostates were dissected free of

their capsules and stored under liquid nitrogen until required. Ten prostates (1.5–2.0 g) were chopped with scissors and homogenised in 18 ml of TEDGM buffer (10 mM Tris, 1 mM EDTA, 1 mM DTT, 10% v/v glycerol and 10 mM sodium molybdate adjusted to pH 7.4 with HCl) in a Potter–Elvehjem homogeniser. The homogenate was centrifuged at 100,000 *g* for 1 h at 4°C to give the cytosol fraction. Protein concentrations were determined by a modification to the method of Lowry.[20]

Assay tubes (in duplicate) contained 1 nM tritiated mibolerone, 1 μM triamcinolone acetonide and a range of concentrations of either "cold" mibolerone (0–100 nM), 4OHA (0.1–100,000 nM) or metabolite (0.1–100,000 nM) in cytosol (200 μl). The tubes were incubated in the presence and absence of 200 nM of "cold" mibolerone at 4°C for 20 h, after which dextan-coated charcoal suspension (0.05% dextran, 0.5% charcoal in TEDGM pH 7.5, 200 μl) was added to separate "free" and "bound" tritiated mibolerone. The tubes were incubated at 4°C for 10 min followed by centrifugation at 11,600 *g* for 2 min. Aliquots of supernatant (0.3 ml) were taken for liquid scintillation counting. "Non-specific" binding is defined as that binding which occurs in the presence of 200 nM of "cold" mibolerone and the specific androgen receptor binding is calculated by subtracting the non-specific binding data from the corresponding data obtained for total binding. The relative binding affinity (RBA) was calculated from the ratio of the concentrations of test compound and "cold" mibolerone required to reduce the specific binding of tritiated mibolerone by 50% (IC_{50}):

$$\text{RBA} = \frac{IC_{50}\ (\text{mibolerone})}{IC_{50}\ (\text{test compound})} \times 100.$$

Preparation of 5α-Reductase from Human Prostate

Human benign prostatic tissue was obtained from patients undergoing radical retropubic prostatectomy. Samples were snap frozen in liquid nitrogen at the time of surgery and then transported to the laboratory for processing. All procedures were carried out at 4°C. Prostatic tissue was minced with scissors and homogenised in two volumes of 50 mM Tris, 1 mM EDTA and 1 mM DTT at pH 7.4. A Polytron homogeniser (setting no. 5 for two 10-s bursts) followed by a Potter Eljvehem homogeniser (2 passes at 2500 rpm) were used. The homogenate was centrifuged at 800 *g* for 10 min. After removing the supernatant, the pellet was resuspended in the same volume of fresh chilled buffer and the centrifugation repeated to derive a crude nuclear preparation. The final pellet was resuspended, snap frozen and stored in liquid nitrogen until used. Protein concentration was determined as described.[20] The 5α-reductase activity was monitored radiometrically by measuring the conversion of [4-^{14}-C] testosterone into [4-^{14}-C]5α-dihydrotestosterone.[21] Each assay tube contained a final substrate concentration of 2.5 μM [4-^{14}C] testosterone (0.1 μCi of [^{14}C]testosterone), 1 mM NADPH, 1 mM DTT, 0.5 nM EDTA and 0.1 M Tris at pH 7.4. For the assays carried out at pH 5.4, the Tris buffer was replaced by 0.1 M succinic acid-sodium hydroxide. Reactions were started by the addition of aliquots of the crude nuclear pellet and incubated at 37°C. The reaction was stopped by the addition of 3 ml ethyl acetate containing 0.2 ml 1 mM mercuric chloride. Precipitated protein was separated by centrifugation at 1500 *g* for 15 min. About 20 μg each of unlabelled testosterone, dihydrotestosterone, androstenedione and 5α-androstan-3β,17β-diol were added to the supernatant which was evaporated to dryness under nitrogen. The steroids were taken up in 40 μl of ethyl acetate and separated by thin-layer chromatography, using dichloromethane : ethyl acetate (9 : 1) as developing solvent. Radioactivity on the

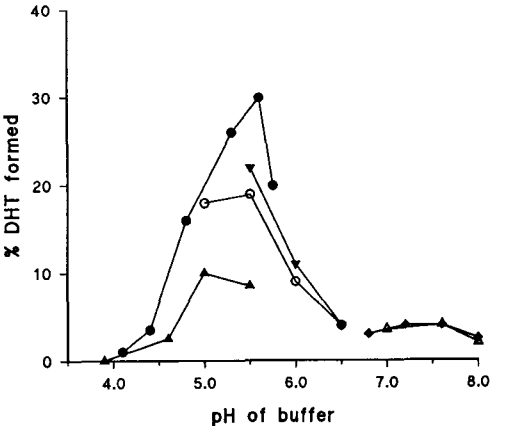

FIGURE 1 Activity of 5α-reductase enzyme (% DHT formed) against buffer pH. All buffers were at a final concentration of 0.1 M and consisted of acetic acid/sodium acetate (▲), succinic acid/NaOH (●), citric acid/trisodium citrate (○), MES/NaOH (▼), HEPES/NaOH (◆) and Tris/HCl (△). The nuclear pellet (1.5 mg protein) was incubated for 30 min and assayed as described. Each point is the mean of triplicate incubations (SD = ±10%).

plate was located and quantified by a Berthold LB283 TLC Linear Analyzer. Non-radioactive steroids were detected by ultra-violet absorption or by exposure of the plate to iodine vapour. The only radioactive product was 5α-dihydrotestosterone (Rf = 0.24) which was well resolved from other steroids (testosterone Rf = 0.13, androstenedione Rf = 0.31, 17β-estradiol Rf = 0.18, estrone Rf = 0.42 and 5α-androstan-3β,17β-diol Rf = 0.09). The quantity of dihydrostestosterone formed during the assay was determined from the percentage of radioactivity at the appropriate Rf value. For inhibition experiments, the assays were run in duplicate with the compounds being added to the assay tubes in dimethyl sulphoxide; control tubes received the same volume of solvent (2% of the final volume). The IC_{50} value is the concentration of inhibitor required to reduce the activity of the enzyme to 50% of its control value at the stated substrate concentration.

RESULTS

Using the assay method described, the crude nuclear pellet and 800 g supernatant fractions of benign prostatic tissue were tested for 5α-reductase activity. Eighty per cent of the enzyme's activity was localised in the crude nuclear pellet. This was used for all further experiments. The graph of pH against 5α-reductase activity (Figure 1) showed the enzyme to have a sharp optimum pH around 5.4, therefore in subsequent experiments all assays were conducted at pH 5.4 using 0.1 M succinic acid–NaOH and repeated at the physiological pH of 7.4 using 0.1 M Tris–HCl. Kinetic analysis using a Lineweaver–Burk plot produced a K_m value of 0.53 μM at pH 7.4 and V_{max} of 0.95 pmol DHT formed per min per mg protein. The respective values at pH 5.4 were 0.50 μM and 14.0 pmol DHT/min/mg. The Lineweaver–Burk plots were linear at both pHs with no evidence of substrate activation or product inhibition. All assays were

TABLE I
Effect of 4OHA and its metabolites on 5α-reductase activity and their binding to the androgen receptor.

Compound	Human 5α-reductase enzyme (IC_{50}, μM)[b]		Rat prostatic androgen receptor (relative binding affinity, RBA)[a]
	pH 7.4	pH 5.4	
4-Hydroxyandrostenedione (4OHA)	15.0	19.0	0.085
4-Hydroxytestosterone (4OHT)	20.0	25.0	75.00
3β,17-Dihydroxy-5α-androstan-4-one (metabolite A)	> 100	> 100	0.485
3α,17-dihydroxy-5β-androstan-4-one-(metabolite B)	> 100	> 100	0.016

[a]For comparison; mibolerone RBA = 100 and dihydrotestosterone RBA = 66.
[b]IC_{50} values were the mean of triplicate determinations (SD = ± 10%).

carried out using a saturating substrate concentration of 2.5 μM and at this concentration activity was linear with time (up to 30 min) and protein concentration (up to 6 mg/ml). 4OHA and 4OHT were weak inhibitors of the human 5α-reductase enzyme with IC_{50} values of 15 and 20 μM, respectively, at pH 7.4 (Table I). Repeating the assays at pH 5.4 led to a significant decrease in potency (4OHA; IC_{50} = 19 μM and 4OHT; IC_{50} = 25 μM). Both the 5α-reduced metabolites were inactive up to the highest concentration assayed, namely 100 μM.

Figure 2 shows the binding curves for mibolerone, 4OHA, 4OHT metabolites A and B to the rat prostatic androgen receptor. The relative binding affinities for all the compounds are shown in Table I. The RBA for 4OHA, 0.085, is very low indicating a weak binding to the androgen receptor, whilst that of 4OHT (RBA = 75) suggests

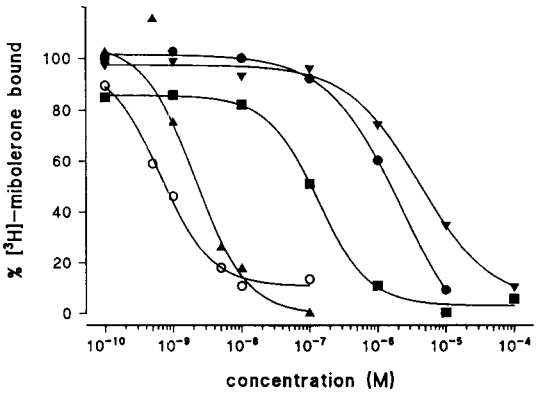

FIGURE 2 Competition of mibolerone, 4OHA, 4OHT and metabolites A & B for the binding of [^{3}H]-mibolerone to the rate prostatic androgen receptor. Cytosols (mean protein concentration = 12.5 mg/ml) were incubated with 1 nM [^{3}H]-mibolerone and increasing concentrations of mibolerone (○) 4OHA (●), 4OHT (▲), metabolite A (■) and metabolite B (▼). Bound and unbound [^{3}H]-mibolerone were separated using dextran coated charcoal and the values corrected for non-specific binding. Each point is the mean of triplicate incubations (SD = ± 10%).

a strong association with the receptor and is comparable to the RBAs of the potent androgens mibolerone and 5α-DHT. Metabolite B (3α,17-dihydroxy-5β-androstan-4-one) is weaker than 4OHA, in comparison metabolite A (3β,17-dihydroxy-5α-androstan-4-one), with a RBA of 0.485, binds about six more times strongly than 4OHA.

DISCUSSION

The localisation of the human prostatic 5α-reductase within the crude nuclear pellet is supported by other studies showing the enzyme to be membrane bound and located in the nuclear fraction, in addition our apparent K_m and V_{max} values, at both pHs, fell within the range of published values.[21,23] The pH optimum of 5.0–5.5 has been reported by others[21,24] and two functional 5α-reductase genes have been identified in man, the major enzyme in prostate tissues displayed an acidic pH optimum, while the other has a more basic pH optimum.[25] The 5α-reductase assays indicate that enzyme activity is weakly inhibited by 4OHA *in vitro*; this is in agreement with results form other groups.[17,26,27] 4OHT has a similar potency towards 5α-reductase as 4OHO, however both the 5α-reduced metabolites are inactive. Detailed endocrine studies on prostatic cancer patients treated with 4OHA have demonstrated that, although estradiol suppression occurred in the majority of patients, the androgen levels, including dihydrotestosterone, either remained constant or showed small increases.[11,12] This suggests that 5α-reductase inhibition by 4OHA and 4OHT is unlikely to be of any clinical significance in these patients.

Our findings that 4OHA binds weakly to the rat androgen receptor supports the observation of 4OHAs weak binding to the androgen receptor of human genital skin fibroblasts.[27] Two metabolites, namely 4OHT and metabolite A are more potent binders (882 and 5.7 times respectively) to the androgen receptor, while metabolite B is weaker than 4OHA. This assay does not distinguish between receptor agonists and antagonists, therefore we cannot directly attribute any androgenic or anti-androgenic effects *in vivo* to a particular metabolite. Both metabolites A and B were detected in urine samples from breast cancer and prostate cancer patients, but 4OHT, as the glucuronide, was only found in the female patients.[13,14] This variation might be due to the different routes of drug administration (oral for female patients and i.m. for males) and/or differences in the metabolism of steroids. However, only urinary metabolites were studied and there is no data so far concerning metabolites in patients' plasma. It is worth noting however that free 4OHT has been detected in the plasma of male rhesus monkeys given 4OHA,[28] so is likely to be present in the plasma of male patients. Women given large oral doses of 4OHA have reduced steroid hormone binding globulin levels suggesting an androgenic effect. However, this effect is small at therapeutic doses and no clinical androgenic symptoms have been observed.[29] The tumour flare seen in male patients was not accompanied by any large increases in serum androgens suggesting the presence of androgenic metabolites.[11,12]

In conclusion, 4OHA and its metabolites are either weak or inactive as human 5α-reductase inhibitors. Although 4OHA binds weakly to the rat androgen receptor, two of its metabolites are more potent binders to the receptor. The ability of these metabolites to bind to the androgen receptor may explain the tumour flare observed in prostate cancer patients treated with 4OHA. In addition, if the binding of the metabolites has an overall anti-androgenic activity, this could provide a mechanism for the clinical benefit derived from 4OHA by some prostatic cancer patients.

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References

1. Ponder, B.A.J., Shearer, R.J., Pocock, R.D., Miller, J., Easton, D., Chilvers, C.E.D., Dowsett, M. and Jeffcoate, S.L. (1984) *Br. J. Cancer*, **50,** 757.
2. Murray, R. and Pitt, P. (1985) *Eur. J. Cancer Clin. Oncol.*, **21,** 453.
3. Drago, J.R., Santen, R.J., Lipton, A., Worgul, T.J., Harvey, H.A., Boucher, A., Manni, A. and Rohner, T.J. (1984) *Cancer*, **53,** 1447.
4. Dowsett, M., Shearer, R.J., Ponder, B.A.J. and Malone, P. (1988) *Br. J. Cancer*, **57,** 190.
5. Plowman, P.N., Perry, L.A. and Chard, T. (1987) *Br. J. Urol.*, **59,** 255.
6. Worgul, T.J., Santen, R.J., Samojilk, E., Veldhuis, J.D., Lipton, A. and Harvey, H.A. (1983) *J. Urol.*, **129,** 51.
7. Coombes, R.C., Goss, P.E., Dowsett, M., Gazet, J-C and Brodie, A.M.H. (1984) *Lancet 2*, 1237–1239.
8. Goss, P.E., Powles, T.J., Dowsett, M., Hutchinson, G., Brodie, A.M.H., Gazet, J-C and Coombes, R.C. (1986) *Cancer Research*, **46,** 4823.
9. Stein, R.C., Dowsett, M., Hedley, A., Davenport, J., Gazet, J-C, Ford, H.T. and Coombes, R.C. (1990) *Cancer Chemother. Pharmacol.*, **26,** 75.
10. Shearer, R.J., Davies, J.H., Dowsett, M., Malone, P.R., Hedley, A., Cunningham, D. and Coombes, R.C. (1990) *Br. J. Cancer*, **62,** 275.
11. Shearer, R.J. (1991) In *Urological Oncology: Dilemmas and Developments "Second-Line Treatment of Advanced Prostate Carcinoma: Aromatase Inhibitors"*. (Alderson, A.R., Oliver, R.T.D., Hanham, I.W.F. and Bloom, H.J.G. (Eds)). p. 243. John Wiley and Sons.
12. Davies, J.H., Dowsett, M., Jacobs, S., Coombes, R.C., Hedley, A. and Shearer, R.J. (1991) *Br. J. Cancer*, in press.
13. Poon, G.K., Jarman, M., McCague, R., Davies, J.H., Heeremans, C.E.M., van der Hoeven, R.A.M., Niessen, W.M.A. and van der Greef, J. (1991) *J. Chromatogr. Biomed. Appl.*, in press.
14. Poon, G.K., Jarman, M., Rowlands, M.G., Dowsett, M. and Firth, J. (1991) *J. Chromatogr. Biomed. Appl.*, **565,** 75.
15. Houston, B. and Habib F. (1988) *Steroids*, **49,** 355.
16. Zoppi, S., Cocconi, M., Lechuga, M.J., Messi, E., Zanisi, M. and Motta, M. (1988) *J. Steroid Biochem.*, **31**(4B), 677.
17. Brodie, A.M.H., Son, C., King, D.A., Meyer, K.M. and Inskster, S.E. (1989) *Cancer Res.*, **49,** 6551.
18. Marsh, D.A., Romanoff, L., Williams, K.I.H., Brodie, H.J. and Brodie, A.M.H. (1982) *Biochem. Pharmacol.*, **31,** 701.
19. Foster, A.B., Jarman, M., Mann, J. and Parr, I.B. (1986) *J. Steroid Biochem.*, **24,** 607.
20. Hartree, E.F. (1972) *Anal. Biochem.*, **48,** 422.
21. Liang, T., Cascieri, M.A., Cheung, A.H., Reynolds, G.F. and Rasmusson, G.H. (1985) *Endocrinol.*, **117,** 571.
22. Houston, B., Chisholm, G.D. and Habib, F.K. (1985) *FEBS Letters*, **185,** 231.
23. Hudson, R.W. (1981) *J. Steroid Biochem.*, **14,** 579.
24. Martin, P.M., LeGoff, J.M., Brisset, J.M., Ojasov, T., Husson, J.M. and Raynaud, J.P. (1987) *Prog. Clin. Biol. Res.*, **243A,** 111.
25. Andersson, S., Berman, D.M., Jenkins, E.P. and Russell, D.W. (1991) *Nature (Lond.)*, **354,** 159.
26. Houston, B. and Habib, F.K. (1988) *Steroids*, **52,** 237.
27. Hsiang, Y-H.H., Berkovitz, G.D., Brown, T.R., Migeon, C.J. and Brodie, A.M.H. (1987) *J. Steroid Biochem.*, **26,** 131.
28. Brodie, A.M.H., Romanoff, L.P. and Williams, K.I.H. (1981) *J. Steroid Biochem.*, **14,** 693.
29. Dowsett, M., Cunningham, D.C., Stein, R.C., Evans, S., DeLennin, L., Hedley, A. and Coombes, R.C. (1989) *Cancer Res.*, **49,** 1306.