Pergamon

0960-0760(94)00145-6

J. Steroid Biochem. Molec. Biol. Vol. 52, No. 1, pp. 71–76, 1995

0960-0760/95 $9.50 + 0.00

The Effect of Aromatase Inhibitor 4-Hydroxyandrostenedione on Steroid Receptors in Hormone-Dependent Tissues of the Rat*

Jiang-Liang Zhou and Angela Brodie†

Department of Pharmacology and Experimental Therapeutics, University of Maryland, School of Medicine, Baltimore, MD 21201, U.S.A.

In previous studies, we observed that aromatase inhibitor 4-hydroxy-androstenedione (4-OHA) treatment significantly decreased estrogen (ER) and progesterone receptor (PR) concentrations in the uterus of ovariectomized (OVX) rats. To investigate whether similar effects occur in mammary tumors, we have studied the hormone-dependent, carcinogen (DMBA)-induced tumors of the rat. After 2 weeks of 4-OHA treatment both ER and PR were reduced in mammary tumors, as well as in uteri of intact animals ($P < 0.05$). Following ovariectomy, receptor levels were also reduced. A further reduction in receptor concentration in mammary tumors occurred with 4-OHA treatment in OVX animals ($P < 0.001$). Treatment of OVX rats with estradiol (0.2 μg/ml) restored tumor PR concentrations to the level of the control, whereas ER levels were increased to concentrations slightly higher than the control. 4-OHA treatment partially inhibited this increase in ER in mammary tumors of OVX rats treated with estradiol. In contrast to ER concentrations, mRNA ER levels in the uterus were not decreased significantly by ovariectomy although mRNA levels were reduced in the tumors. Ovariectomy was without effect on mRNA PR in either tissue. Treatment with 4-OHA reduced mRNA levels of ER and PR in uterus and tumors in intact and OVX animals. Levels of tumor mRNA of both ER and PR were inhibited by 4-OHA treatment in estradiol treated OVX rats. Thus, 4-OHA appears to inhibit ER and PR concentrations in mammary tumors of the rat by reducing transcription. Although aromatase inhibition which results in decreased estrogen production, is the major antitumor effect of 4-OHA, reduction in ER and PR could contribute to effective estrogen blockade and limit tumor growth by antagonizing estrogen action as well as production.

J. Steroid Biochem. Molec. Biol., Vol. 52, No. 1, pp. 71–76, 1995

INTRODUCTION

We have previously shown that 4-hydroxyandrostenedione (4-OHA) is a potent and selective inhibitor of aromatase which also appears to cause inactivation of the enzyme [1, 2]. Further studies in animals [1, 3] and humans demonstrated that this compound is effective in reducing estrogen concentrations *in vivo* and in causing regression of breast carcinoma in postmenopausal patients [4, 5].

We have also investigated whether 4-OHA possesses hormonal activities, in addition to aromatase inhibition, which may contribute to its effects during *in vivo* treatment. No direct interaction of the compound with either the estrogen (ER) or progesterone receptor (PR) was found to occur *in vitro* even at high concentrations [6], although weak androgenic activity (< 1% of testosterone) was detected [1]. However, we subsequently observed that 4-OHA treatment of ovariectomized (OVX) rats, significantly decreased the concentration of the uterine ER and also of the PR, a protein induced by estrogen action [6]. Reduction in steroid concentrations in the tumor as well as inhibition of aromatase activity could provide highly effective blockade of estrogen stimulated tumor growth. Therefore, in the

*This study was presented in part at the 1990 *Annual Meeting of the Endocrine Society*.
†Correspondence to A. Brodie.
Received 20 June 1994; accepted 23 Aug. 1994.

study reported here, we have investigated the effect of 4-OHA treatment on steroid receptor levels in mammary tumors of the rat. In addition, we sought to determine whether the reduction in steroid receptors in hormone responsive tissues is mediated via alterations in transcription and have measured the mRNA levels of ER and PR.

MATERIALS AND METHODS

Animals

Sprague–Dawley female rats of 220–250 g (designated "normal" rats) and rats aged 45–50 days were purchased from Harlan Breeding Laboratories, Chicago, IL. The latter animals were gavaged with 20 mg 7,12-dimethylbenz(a)anthracene (DMBA) (Sigma) in 2 ml peanut oil under light anesthesia between 50–55 days of age and are designated "DMBA" rats. All animals were housed under controlled conditions of light (12 h light–dark), temperature and humidity and had access to food and water *ad libitum*. Approximately two months after DMBA administration, tumors began to develop. They were measured weekly with calipers and their volumes calculated as previously [1]. Body weights of the rats were also measured weekly. When one tumor per rat reached at least 2 cm diameter, the animal was assigned to a treatment group, usually five per group. Since the number of tumors and their volumes varied from rat to rat, the treatment groups were balanced so that each contained similar numbers of animals with comparable total volumes and numbers of tumors.

Treatments were prepared as suspensions in 0.3% hydroxypropylcellulose (Sigma) and were injected s.c. daily for 2 weeks. At the end of the treatment period, the rats were anesthetized, tumors measured and removed, uteri collected and then the animals were euthanized. The tissues were cleaned of fat and necrotic areas, weighed and stored at −70°C until assayed.

Steroid receptor assays

Steroid receptor concentrations were measured as described previously [6] and were analyzed by the method of Scatchard [7] using at least six estimates per sample. Briefly, frozen pooled uteri and tumors were pulverized and then homogenized in TEDG buffer (Tris–HCl, 10 mM, pH 7.5, EDTA, 2 mM, β-mercaptoethanol), centrifuged at 4200*g* for 15 min and the supernatant centrifuged at 105,000*g* for 1 h at 4°C. Aliquots of the supernatants were incubated overnight at 4°C with either [2,4,6,7,17β-^{3}H]estradiol (93 Ci/mmol) or with [17α-methyl-^{3}H]promgesterone (R5020) (87 Ci/mmol) (New England Nuclear, Boston, MA) in the presence or absence of varying concentrations of unlabelled estradiol or R5020, respectively. Non-specific binding was determined by the addition of 100-fold excess of DES or R5020. Unbound steroids were removed by incubating with dextran-coated charcoal for 15 min at 4°C followed by centrifugation at 8000*g*. The amount of receptor-bound labelled steroid in the supernatant was determined by liquid scintillation counting. Tissue protein concentrations were determined by the method of Lowry [8] and ER and PR concentrations expressed as fmol/mg protein.

Measurement of estrogen and progesterone mRNA

Total RNA was extracted using the method of Chirgwin *et al.* [9]. Frozen tissues were pulverized under liquid nitrogen, then homogenized in ice-cold 4 M guanidine thiocyanate (GT) buffer (12 ml/g of tissue). The homogenates were centrifuged at 8000*g* for 10 min at 4°C to eliminate particulate material. RNA was sedimented by ultracentrifugation (18 h at 174,000*g*/32,000 rpm, 20°C) through a cesium chloride gradient (5.7 M $CsCl_2$, 100 mM EDTA). The pellets were washed with ice cold 95% ethanol, and dissolved in 400 μl STE buffer (10 mM Tris, 0.1% SDS, 5 mM EDTA, pH 7.4). The RNA was precipitated by adding 40 μl of 3 M sodium acetate (pH 6) and 2.5 vol. of 95% ethanol, and incubating for 1 h at −20°C. Following centrifugation (15 min at 10,000*g*), the pellets were dried and dissolved in water. The concentration of RNA was determined by UV absorbance at 260 nm. Poly(A) + RNA was isolated by oligo (dT)-cellulose column chromatography and then was subjected to agarose gel electrophoresis 40 V for 5 h at room temperature using glyoxal buffer. RNA was capillary transferred by capillary action to Greenscreen nylon filters (New England Research Products, Boston, MA) (24 h at room temperature) and then hybridized with ^{32}P-labelled cDNA probes for ER and β-actin at 42°C overnight [10]. After hybridization, filters were washed under high stringency conditions. Filters were exposed overnight to X-ray (Kodak) film at −70°C and the intensity of the areas measured by densitometry. The filters were then extensively washed and rehybridized with the PR probe. ER cDNA was kindly provided by the late Dr W. McGuire, San Antonio, TX and the PR cDNA by Dr B. O'Malley, Houston, TX.

RESULTS

The effect of treatments on uterine estrogen and progesterone receptors

Treatment of normal intact rats with 4-OHA (50 mg/kg/day) caused significant reduction in both uterine ER and PR concentrations but no change in the receptor binding affinity (apparent K_d) (data not shown). Administration of 4-OHA in doses ranging from 0.1–50 mg/kg/day to five rats per group produced dose-related reductions in cytosolic ER and PR concentrations, in rat uteri compared to control rats ($n = 7$) (Fig. 1). The receptor levels were reduced more than 50% by 50 mg/kg/day 4-OHA in three separate experiments ($P < 0.05$). When rats were ovariectomized, uterine ER and PR levels were also reduced ($P < 0.05$).

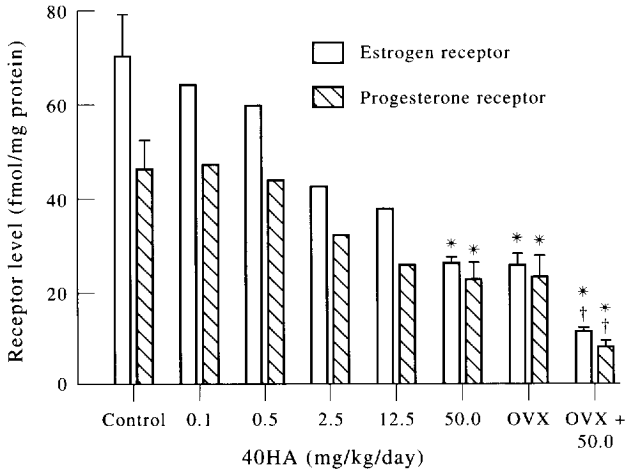

Fig. 1. The effects of 4-OHA and/or ovariectomy on cytosolic ER and PR concentrations in the rat uterus. Groups of 5 normal rats (without tumors) were injected s.c. daily with 0.1–50 mg/kg 4-OHA, ovariectomized, or ovariectomized and treated with 4-OHA (50 mg/kg). After 2 weeks, uteri were removed and assayed for cytosolic steroid receptor concentrations by Scatchard analysis as described under Methods. Mean values ± SE are separate experiments; *$P < 0.05$ vs control group; †$P < 0.05$ vs OVX rats.

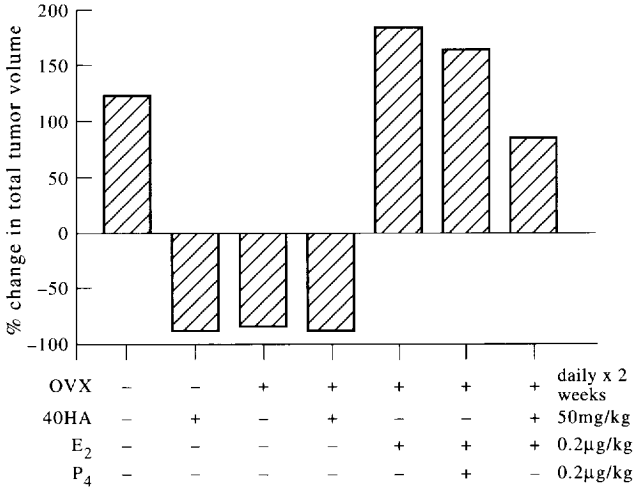

Fig. 2. The effects of ovariectomy and/or treatment with various steroids on the growth of DMBA-induced mammary tumors of the rat. Groups of rats with at least one tumor of 2 cm diameter each were injected s.c. daily for 2 weeks with vehicle ($n = 4$ rats, 8 tumors initially) or 4-OHA (50 mg/kg) ($n = 6$ rats, 10 tumors), or were ovariectomized ($n = 5$ rats, 5 tumors) and treated with vehicle, 4-OHA (50 mg/kg) ($n = 5$ rats, 5 tumors), estradiol (E_2) (0.2 μg/kg) ($n = 5$ rats, 6 tumors) or progesterone (P_4) (0.2 μg/kg) (5 rats, 5 tumors) or combinations of these steroids. The percentage change in total tumor volume of each group measured after 2 weeks of treatment relative to their initial volumes is shown.

A further reduction in receptor concentrations occurred when OVX animals were treated with 4-OHA (50 mg/kg) ($P < 0.05$).

The effect of treatments on mammary tumor volumes

In rats bearing DMBA-induced, hormone dependent mammary tumors, ovariectomy or treatment with 4-OHA caused marked regression in tumor volumes to approximately the same extent. However, there was a large variation in tumor size following ovariectomy; most tumors decreased but some increased in volume. In contrast, treatment with 4-OHA produced a consistent reduction in volume. Tumor growth resumed in OVX rats treated with either estradiol, or estradiol and progesterone. At the dose of estradiol used, tumor volumes were increased more than in control rats after 2 weeks of treatment. Treatment with 4-OHA was partially effective in reducing estrogen stimulated growth in OVX rats (Fig. 2).

Estrogen and progesterone receptors in mammary tumors

There was a significant decrease in ER and PR concentrations in the tumor tissue from 4-OHA treated rats and OVX animals (Fig. 3). Treatment of OVX rats with 4-OHA produced a further significant reduction in tumor ER concentrations from 69.6 ± 5.6 to 21.8 ± 2.7 fmol/mg protein ($P < 0.001$). The PR concentrations were similarly reduced from 57.0 ± 2.5 fmol/mg protein in OVX rats to 23.4 ± 3.1 fmol/mg protein in OVX rats treated with 4-OHA ($P < 0.001$). When OVX animals were injected with estradiol or with estradiol plus progesterone (0.2 μg/kg of each), ER concentrations were induced to levels above those of intact rats, whereas PR concentrations were restored to approximately the level of the intact controls. Co-administration of 4-OHA with estradiol, counteracted the effects of estradiol on ER but had a smaller effect on PR, reducing the level by about 25%.

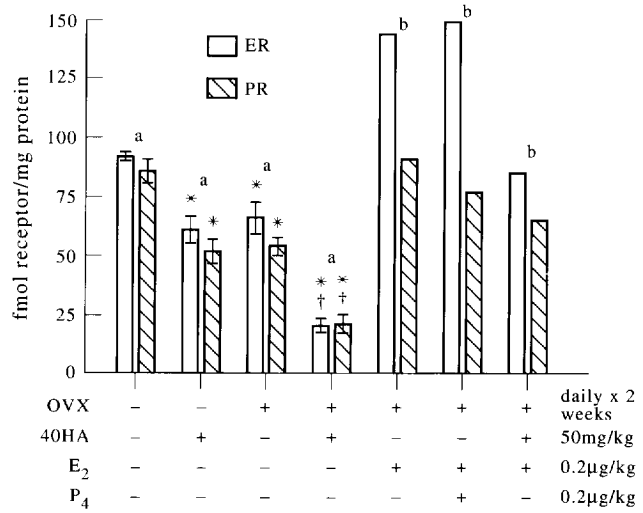

Fig. 3. The effects of ovariectomy and/or treatment with various steroids on cytosolic ER and PR concentrations in DMBA-induced mammary tumors of the rat. Estrogen receptor and progesterone receptor concentrations were measured in mammary tumors from the same animals as in Fig. 2. (a) Data (× ± SEM) derived from Scatchard analysis of ligand binding data, $n = 6$ tumors from 5 individual rats; *$P < 0.05$ vs control; †$P < 0.001$ vs OVX. (b) Scatchard analysis from tumor tissue pooled from 5 rats.

mRNA for estrogen and progesterone receptors

Measurements of mRNA concentrations for ER and PR were next compared in both uterine and tumor tissue from DMBA rats to determine the effects of 4-OHA on transcription of these receptors. Figure 4(A and B) show representative data of several repeat experiments for which the overall conclusions were consistent. Treatment of intact DMBA rats with 4-OHA caused decreases of at least 50% in mRNA concentrations for both ER and PR in the uterus and also in the tumors. In contrast to its effect on steroid receptor concentrations, ovariectomy did not reduce mRNA levels of PR in the uterus and produced only a small decrease of 15–20% in the mRNA PR concentrations of tumors. Furthermore, the concentration of mRNA ER was only slightly reduced in the uterus by ovariectomy, although it declined by 60% in mammary tumors. Treatment of OVX rats with 4-OHA further reduced mRNA levels of both ER and PR in the uterus and tumors. Administration of estradiol to OVX animals fully restored mRNA levels for ER in uterine and tumor tissue to intact control values. Levels of mRNA PR which were only slightly reduced in mammary tumors by ovariectomy were also restored to intact levels. Co-injections of 4-OHA with estradiol in OVX, DMBA-treated rats reduced mRNA concentrations for ER by approx. 50% and mRNA PR by 75% in tumor tissue compared to the effects of estradiol alone. There were no significant changes in β-actin mRNA concentrations which were measured in all tissue as controls.

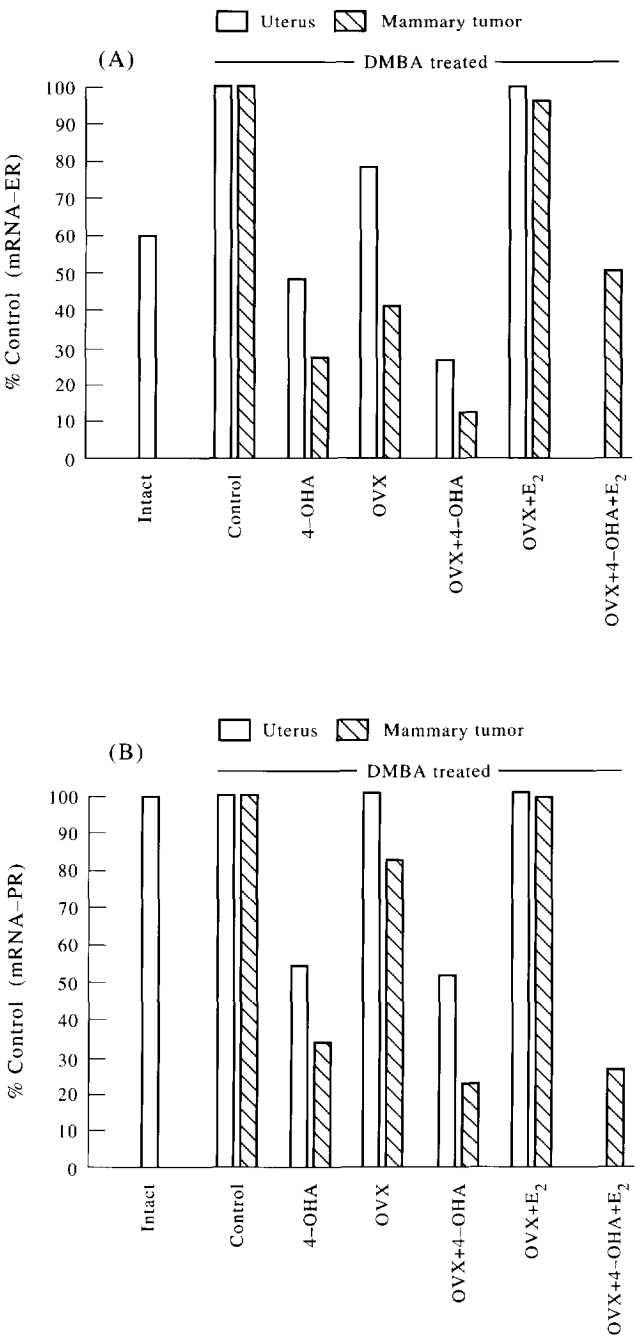

Fig. 4. The effects of ovariectomy and/or treatment with 4-OHA or estradiol on concentration of mRNA ER (A) and PR (B) in uteri and DMBA-induced mammary tumors of the rat. Groups of animals with at least one tumor of 2 cm diameter each, were injected s.c. daily for 2 weeks with vehicle or 4-OHA (50 mg/kg), or were ovariectomized and treated with vehicle, 4-OHA (50 mg/kg), estradiol (E_2) (0.2 μg/kg) or both 4-OHA and estradiol. Densitometric measurement of a representative of several repeat Northern analysis of mRNA ER and PR in the uteri and tumors as described in Methods.

DISCUSSION

Results of the current study confirm our previous findings that treatment of OVX rats for 2 weeks with 4-OHA decreased both uterine ER and PR concentrations [6]. In addition, we observed a dose–response effect in intact rats between 0.1–50 mg/kg with an approximate reduction in ER and PR of 50% occurring at 50 mg/kg 4-OHA. No dose effect was observed with doses greater than 12.5 mg/kg/day (data not shown). While decreased receptor levels in intact rats may be due to reduction in estrogen concentrations via inhibition of ovarian estrogen synthesis by 4-OHA, this explanation cannot account for the reduction in receptor concentrations in OVX animals.

In our previous study, we also found that antiestrogen treatment increased PR levels in the rat uterus, an effect blocked by co-administration of 4-OHA [6]. This result suggested that 4-OHA may antagonize the estrogenic action of the antiestrogen *in vivo*. As 4-OHA is now being used in the treatment of breast cancer patients [7], we considered it important to determine whether there are similar effects of 4-OHA on steroid receptors in mammary tumors. In the current studies, we have utilized the carcinogen-induced (DMBA), hormone-dependent, mammary tumor model which we have previously employed to investigate the effects of 4-OHA on tumor growth [1, 3, 6]. Since there is great variability in the initial tumor volumes, we express

results as percentage change in total tumor volume [3] rather than mean values. Most tumors regress almost completely during 4 weeks of treatment with 4-OHA. Therefore, tumors were removed for study after 2 weeks, at which time significant amounts of viable tissue remain. Similar reductions in ER and PR concentrations were found to occur in mammary tumor tissue as in the uterus of rats treated with 4-OHA for 2 weeks ($P < 0.05$). For comparison with 4-OHA treatment, other rats were ovariectomized. Surgical removal of ovarian estrogen sources also caused reductions in ER and PR concentrations which could be counteracted by estradiol injections. However, when OVX rats were treated with 4-OHA, steroid receptors concentrations were further reduced. Moreover, 4-OHA partially inhibited the increase in ER concentrations in OVX rats treated with estradiol. This suggests a mechanism of the compound which involves antagonism of estrogen action and may explain the reduction in tumor growth when estrogen stimulated OVX rats are treated with 4-OHA. Thus, it appears that these actions of 4-OHA are independent of its inhibition of aromatase and estrogen synthesis. We therefore investigated whether the effects of 4-OHA on receptor concentrations are mediated by regulation of transcription of these proteins.

Interestingly, ovariectomy did not cause a fall in mRNA levels for uterine PR and only slight reductions in mRNA PR in tumors. The mRNA ER concentration in the uterus was reduced by only a small extent and not below the level in the intact rat uterus [Fig. 3(a)]. In contrast, mRNA levels in the tumors were reduced by 50% after ovariectomy. It is possible that the reduction in receptor protein concentrations observed in rats 2 weeks after ovariectomy, may be due in part to increased degradation of the receptor protein occurring in the absence of ligand rather than an effect on transcription. Saceda *et al.* have reported that the ER is unstable in the absence of estradiol [11]. Treatment with 4-OHA (Fig. 3), resulted in marked reduction in mRNA ER levels in intact animals and an even greater reduction in OVX animals. This might occur if the half-life of the mRNA and/or its rate of transcription was decreased. Autologous down-regulation is a prevalent finding among steroid receptor mRNA [12, 13]. In the current situation, it appears that heterologous regulation may be occurring. There are a few reports of other steroids affecting different receptor concentrations [14] but this is the first report investigating effects on transcription. The above results suggest that 4-OHA may down regulate ER and PR by its effects on transcription.

Although some of the effects of 4-OHA appear to be similar to those of DHT [6], further studies are needed to determine whether these actions on ER and PR transcription are mediated via the androgen receptor. 4-OHA is converted *in vivo* to 4-hydroxytestosterone (4-OHT) and 3β-hydroxy-5-androstan-4,17-dione which are its two most important metabolites [15, 16]. 4-OHT also inhibits aromatase but is less potent (60%) than 4-OHA. We have previously shown that both of these metabolites are weakly androgenic and bind to the androgen receptor and thus could be responsible for these effects. The androgenic activity of 4-OHT and other metabolites was also recently reported by Davis *et al.* [17].

Although aromatase inhibition is the major action of 4-OHA, reduction in ER concentrations could contribute to effective estrogen blockade and limit tumor growth by antagonizing estrogen action as well as production.

Acknowledgement—This work was supported by NIH grant HD-13949.

REFERENCES

1. Brodie A. M. H., Schwarzel W. C., Shaikh A. A. and Brodie H. J.: The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. *Endocrinology* 100 (1977) 1684–1695.
2. Brodie A. M. H., Garrett W. M., Hendrickson J. R., Marcotte P. A. and Robinson C. H.: Inactivation of aromatase activity in placental and ovarian microsomes by 4-hydroxyandrostene-3,17-dione and 4-acetoxyandrostene-3,17-dione. *Steroids* 38 (1981) 693–702.
3. Brodie A. M. H., Garrett W. M., Hendrickson J. R. and Tsai-Morris C.-H.: Effects of 4-hydroxyandrostenedione and other compounds in the DMBA breast carcinoma model. *Cancer Res.* 42 (1982) 3360s–3364s.
4. Coombes R. C., Goss P. E., Dowsett M., Hutchinson G., Cunningham D., Jarman M. and Brodie A. M. H.: 4-Hydroxyandrostenedione treatment for postmenopausal patients with advanced breast cancer. *Steroids* 50 (1987) 245–252.
5. Goss P. E., Powles T. J., Dowsett M., Hutchinson G., Brodie A. M. H., Gazet J. C. and Coombes R. C.: Treatment of advanced postmenopausal breast cancer with an aromatase inhibitor 4-hydroxyandrostenedione: Phase II report. *Cancer Res.* 46 (1986) 823–4826.
6. Wing L.-Y. C., Hammond J. O. and Brodie A. M. H.: Differential responses of sex steroid target tissues of rats treated with 4-hydroxyandrostenedione. *Endocrinology* 122 (1988) 2418–2427.
7. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51 (1949) 660–672.
8. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. L.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193 (1951) 265–275.
9. Chirgwin J. M., Pryzbyla A. E., MacDonald R. J. and Rutter W. J.: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18 (1979) 5295–5299.
10. Sambrook J., Fritsch E. F. and Maniatis T.: *Molecular Cloning: A Laboratory Manual.* 2nd Edn. Cold Spring Harbor Laboratory Press (1989).
11. Saceda M., Lippman M. E., Chambon P., Lindsey R. L., Ponglikitmongkol M., Puente M. and Martin M. B.: Regulation of the estrogen receptor in MCF-7 cells by estradiol. *Molec. Endocrinology* 2 (1988) 1157–1162.
12. Quarmby V. E., Yarbrough W. G., Lubahn D. B., French F. S. and Wilson E. M.: Autologous down-regulation of androgen receptor messenger ribonucleic acid. *Molec. Endocrinology* 4 (1990) 22–28.
13. Wei L. L., Krett N. L., Francis M. D., Gordon D. F., Wood W. M., O'Malley B. W. and Horwitz K. B.: Multiple human progesterone receptor messenger ribonucleic acids and their autoregulation by progestin agonists and antagonists in breast cancer cells. *Molec. Endocrinology* 2 (1988) 62–72.

14. Stover E. P., Krishnan A. V. and Feldman D.: Estrogen down regulation of androgen receptors in cultured human mammary cancer cells (MCF-7). *Endocrinology* **120** (1987) 2597–2603.
15. Marsh D. A., Romanoff L. P., Williams K. I. H. and Brodie A. M. H.: Synthesis of deuterium and tritium labelled 4-hydroxyandrostenedione, an aromatase inhibitor, and its metabolism *in vitro*. *Biochem. Pharmac.* **31** (1982) 701–705.
16. Jarman M.: Pharmacokinetic and metabolic studies with 4-hydroxyandrostenedione. In *4-Hydroxyandrostenedione—A New Approach to Hormone-Dependent Cancer*. (Edited by Coombes and Dowsett) Royal Soc. Med. Services Ltd., London (1991) pp. 13–18.
17. Davis J. H., Shearer R. J., Rowlands M. G., Poon G. K., Houghton J., Jarman M. and Dowsett M.: Effects of 4-OHA and its metabolites on 5α-reductase activity and the androgen receptor. *J. Enzyme Inhibition* **6** (1993) 141–148.