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EFFECT OF AN AROMATASE INHIBITOR, 1,4,6-ANDROSTATRIENE-3,17-DIONE, ON 7,12-DIMETHYLBENZ[*a*]ANTHRACENE-INDUCED MAMMARY TUMORS IN THE RAT AND ITS MECHANISM OF ACTION *IN VIVO**

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Abstract—In this study, 1,4,6-androstatriene-3,17-dione (ATD) was demonstrated to cause time-dependent loss of aromatase activity in rat ovarian microsomes *in vitro*. *In vivo*, an injection of ATD caused inhibition of ovarian aromatase and reduced estrogen secretion in pregnant mare's serum gonadotropin-primed rats for at least 24 hr after injection. In rats with 7,12-dimethylbenz-[*a*]anthracene-induced, hormone-dependent, mammary tumors, marked regression occurred with ATD treatment. Although estrogen secretion was not reduced below the diestrus level of controls, the rats remained anestrus, indicating that the proestrus surge of estrogen was prevented. LH, FSH and prolactin levels were also basal and LH and FSH did not rise after ovariectomy. ATD had no detectable hormonal activity in bioassay. Consistent with this, the compound did not interact appreciably with either androgen or estrogen receptors, was not uterotrophic, and did not interfere with mammary tumor regression in ovariectomized rats. Thus, the major activities of the compound which cause mammary regression in the rat appear to be inhibition of estrogen synthesis, via aromatase and gonadotropin suppression.

The biosynthesis of estrogens involves the aromatization of androgens to estrogens. This is a unique reaction in steroid biosynthesis; also, it is the last step in the biosynthetic sequence from cholesterol to estrogens. Therefore, compounds inhibiting the enzyme system mediating aromatization (aromatase) would be expected to be more specific for estrogen biosynthesis than inhibitors of enzymes influencing earlier steps in steroidogenesis.

Inhibition of estrogen production by aromatase inhibitors might be an effective means of treating patients with estrogen-dependent tumors, as in breast and endometrial cancer. Approximately, a third of breast cancer patients have hormone-dependent tumors. Deprivation of estrogens in such patients results in tumor regression which may often be long-lasting. Traditionally, ablation of estrogens was performed by ovariectomy. However, since aromatization occurs not only in the ovaries but also in peripheral tissues [1, 2] and some breast tumors [3–6], aromatase inhibitors could be more effective in preventing estrogen production and possibly less traumatic than surgical methods.

We have shown previously that aromatase inhibitors, 4-hydroxyandrostene-3,17-dione (4-OHA) and 4-acetoxyandrostene-3,17-dione (4-acetoxyA), reduce ovarian estrogen secretion [7, 8], inhibit peripheral aromatization to estradiol and estrone in the primate [9], and are highly effective in causing tumor regression of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced, hormone-dependent, mammary tumors in the rat [7, 8].

In this report, we evaluated the effectiveness of 1,4,6-androstatriene-3,17-dione (ATD), also a potent aromatase inhibitor [10–12] in causing regression of DMBA-induced mammary tumors. In addition, we describe studies of other mechanisms which may contribute to the effectiveness of this compound *in vivo* and compare them with those of pharmacological doses of androgens and estrogens.

MATERIALS AND METHODS

1,4,6-*Androstatriene*-3,17-*dione*

ATD was obtained from Steraloids and purified as described [12].

In vitro *inhibition of ovarian aromatase by ATD*

Ovarian microsomes obtained from rats treated for 12 days with s.c. injections of 100 I.U. of pregnant mare's serum gonadotropin (PMSG) on alternate days were prepared as previously [11]. The microsomes (0.8 mg protein per incubation) were preincubated at 37° with 1 and 5 μM ATD and NADPH. After various times (0–75 min) 1 ml (0.5%) of char-

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coal was added to each incubation to remove ATD. Following centrifugation (1500 *g*), the supernatant fraction was incubated with [1,2-^{3}H]androstenedione (200,000 dpm) and NADPH for 30 min at 37°. The amount of tritium released into the medium as tritiated water during aromatization was taken as a measure of aromatase activity after extraction of steroids with chloroform [13, 14].

Ovarian microsomes were also incubated for 1 hr as above, after which the microsomal pellet was precipitated by ultracentrifugation. The pellet was resuspended in buffer, diluted, resuspended a second time, and then allowed to stand at 0° overnight. The amounts of protein and aromatase activity were assayed after the first centrifugation and after standing overnight.

In vivo *inhibition of ovarian aromatase by ATD treatment*

Rats, primed with PMSG as above, were injected on day 12 with 50 mg/kg ATD in Steroid Suspending Vehicle: Klucel (5:1) (supplied under the auspices of the National Cancer Institute). After 8 hr, ovarian-vein blood for estrogen determinations was collected from two treated rats and one control rat (vehicle injected). Microsomes then were prepared from the ovaries, and the aromatase activity was measured. These procedures were repeated 24 hr after ATD injection with two treated rats and one control rat, and after 48 hr with two treated and two control rats.

Mammary tumor studies

Animal studies were carried out as described previously [7]. In brief, 50- to 55-day-old female rats of the Sprague–Dawley strain (Charles River Breeding Laboratories, Cambridge, MA) were gavaged with 20 mg DMBA in 2 ml peanut oil. Animals were selected for experiments when at least one tumor per rat had reached 2 cm diameter (measured with calipers). Each group consisted of rats with approximately the same total number of tumors and total tumor volume (tumor volume of each tumor was calculated as $v = 4/3\pi r_1^2 r_2$, where r_1 is the minor radius [15]).

The rats were implanted with silastic wafers prepared from 0.5 g silastic (medical grade elastomer 382, Dow Corning) with (or without, for controls) 150 mg ATD per wafer [12]. The silastic disc was divided into four pieces and each was inserted under the dorsal skin. Each rat was injected twice daily with 12.5 mg/kg ATD. Control animals were injected with vehicle. Vaginal smears were made daily. Each week the animals were weighed, tumors were measured, and the silastic implants were replaced. Used implants were weighed and the ATD was extracted with ether in a Soxhlet extractor for 3 hr. The ether was evaporated and the amount of ATD, dissolved in methanol, was quantitated by ultraviolet absorption.

Experiment 1. A group of eight rats with a total of twenty-eight tumors was treated with ATD and a group of seven rats served as controls (see Fig. 3). After 4 weeks, 3 ml of blood was collected from the carotid artery of each rat. Since the treated animals remained anestrus throughout, judged by daily vaginal smears, control rats were bled on diestrus I. Gonadotropin (LH, FSH) and prolactin levels (Table 2) were assayed by the procedures of Odell *et al.* [16].

The silastic implants remained in place for another 3 weeks and twice daily injections were continued, a total of 7 weeks of treatment. Control animals were in poor condition by this time and were killed. The ATD-containing implants were removed from the treated animals, injections were stopped, and the rats were observed for 7 weeks. Five of the rats were then treated with ATD as before for 5 weeks. At the end of this time, two of these rats had tumors remaining and were ovariadrenalectomized and observed for 4 more weeks.

Experiment 2. Two groups of six rats were treated as above. In addition, one group also received daily s.c. injections ($0.3\ \mu g \cdot kg^{-1} \cdot day^{-1}$) of estradiol. A third group injected with vehicle served as control animals. Tumors were measured weekly for 4 weeks (Fig. 4). Ovarian-vein blood was collected on the last day of treatment to determine estradiol secretion.

Experiment 3. Groups of five rats with mammary tumors were ovariectomized and then treated with one of the following regimens: ATD administered as above, testosterone $20\ mg \cdot kg^{-1} \cdot day^{-1}$, or estradiol $0.3\ \mu g \cdot kg^{-1} \cdot day^{-1}$. Two groups of intact rats treated with either testosterone or ATD were compared. Tumor volumes were measured weekly as before for 4 weeks (Table 3).

The effects of these treatments on LH, FSH and prolactin levels were also studied. For this experiment, groups of eight normal cycling rats were ovariectomized and treated from the day of surgery with ATD, testosterone or estradiol ($0.3\ mg \cdot kg^{-1} \cdot day^{-1}$) as above for 8 days. Blood was collected following decapitation and the plasma levels of gonadotropins were assayed by radioimmunoassay [16]. Uterine wet weights were also measured.

Studies of ATD competition for steroid binding sites

Estrogen receptor. To determine whether ATD interacts with estrogen receptors, the compound was incubated with rat uterine cytosolic and nuclear fractions.

Uteri from immature rats (19–21 days) were incubated with [^{3}H]estradiol and ATD, testosterone, or estradiol for 1 hr at 37° under 95% O_2/5% CO_2. ATD and testosterone were prepared in ethanol at concentrations of 10^{-5} M, 10^{-7} M, and 10^{-9} M, while estradiol concentrations were 10^{-6} M, 10^{-8} M, and 10^{-10} M. After incubation, the nuclear and cytosolic fractions were prepared according to the method of Clark *et al.* [17, 18], and estradiol receptor sites were estimated.

Cytosolic androgen receptor binding. Cytosol from levator ani muscles from 4-day gonadectomized male rats were used in this dextran–charcoal assay [19, 20]. R1881-[^{3}H] (methyltrienolone) was the radioligand, as it binds selectively to the androgen receptor, is not metabolized, and is not bound to serum proteins. One hundred-fold excess of R1881 was used for measurement of nonspecific binding and 500-fold molar excess of triamcinolone acetonide was added