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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

to all tubes to prevent possible binding of R1881 to a progestin receptor.

Competition studies were carried out with labeled R1881 at 210 nM (i.e. saturating concentration) by comparing specific binding in the presence and absence of ATD (10^{-5}–10^{-7} M).

RESULTS

Aromatase inhibition with ATD

Preincubation of microsomes with ATD and NADPH caused time-dependent loss of aromatase activity, as shown in Fig. 1. This effect was not observed in the absence of NADPH. When the microsomes were allowed to stand overnight at 4° after removal of ATD with charcoal and washing, and further washing the following morning, no recovery of aromatase activity occurred. Loss of aromatase activity also was observed in the ovarian microsomes prepared from rats given an injection of ATD and killed at various times. The activity declined from 8 to 24 hr after injection but returned almost to control levels by 48 hr (Fig. 2). The decline in ovarian secretion of estrogen followed a pattern similar to the loss of aromatase activity, although the decrease in estrogen secretion was not as great as the decrease in enzyme activity. However, the control output of estrogen was greatly stimulated by PMSG and remained relatively constant from 0 to 48 hr, 3.65 ± 0.63 (S.E.) ng/ml.

Effect on ATD on tumor progression

The mean release rate of ATD from the silastic implants was $10.5\ mg \cdot day^{-1} \cdot (300\ g\ rat)^{-1}$.

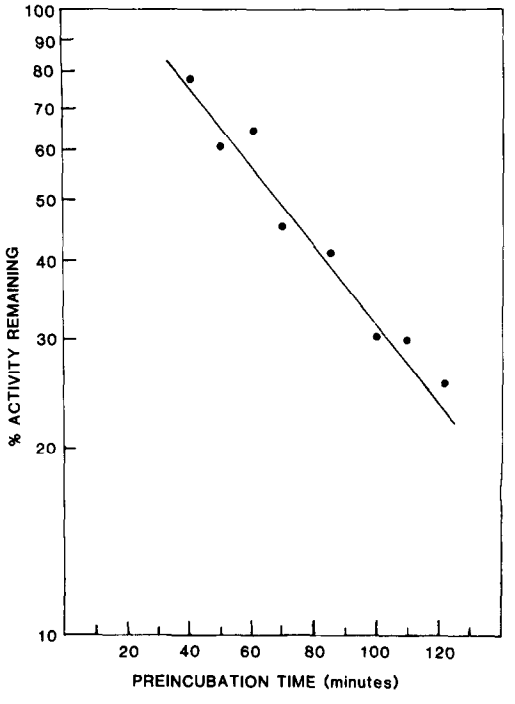

Fig. 1. Aromatase activity of PMSG-primed rat ovarian microsomes on preincubation with ATD (5 μM) and 1 mM NADPH at 37°.

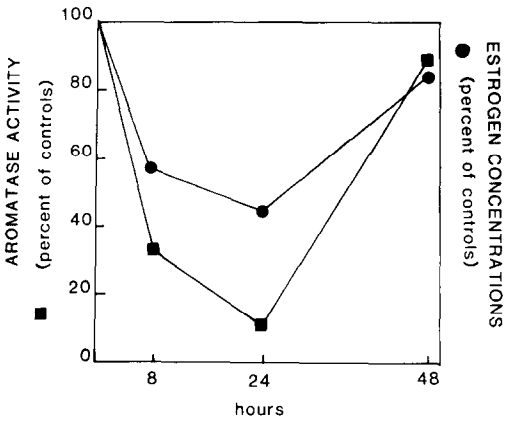

Fig. 2. Aromatase activity (■) (% of controls) of ovarian microsomes and estrogen concentrations (●) in ovarian-vein plasma from the same PMSG-primed rats autopsied at various times after s.c. injection of ATD (50 mg/kg) at 0 time.

Together with the amount injected, the rats received a total dose of about $50\ mg \cdot kg^{-1} \cdot day^{-1}$, similar to the dose used for other aromatase inhibitors [7].

ATD was highly effective in causing significant tumor regression of 96% in 4 weeks in both experiments 1 and 2 (Figs. 3 and 4). Values for ovarian estrogen secretion for ATD-treated rats in experiment 2 were not significantly reduced below the values for the control rats bled during diestrus. However, the treated animals remained anestrus as judged by daily vaginal smears. Our previous data demonstrated that ATD inhibited the proestrus surge and prevented ovulation [12]. In the present studies, the addition of estradiol to ATD treatment prevented tumor regression observed with ATD alone (Fig. 4). Thus, it would seem that although estrogen secretion was reduced during the cycle, it was not entirely eliminated by ATD treatment. However, as can be seen in Figs. 3 and 4, extensive tumor regression occurred in 3–4 weeks. After a further 3 weeks of treatment (Fig. 3), there was little change in the total tumor volume, because of the growth of one tumor during that time. All other tumors regressed to a size too small to measure and could only be palpated, or regressed completely. Thus, in 7 weeks the total eliminated was 59% (Table 1). By comparison, in the seven control rats with twenty-one tumors, 33% regressed significantly but 48% of the tumors more than doubled in size in 7 weeks.

ATD treatment was not administered to the rats for the next 7 weeks. By the end of that time, ten of the seventeen tumors that had completely regressed, reappeared. In addition, thirteen new tumors grew. When a second course of treatment with ATD was administered, by the same method as before, to five of the eight rats for 5 weeks (Fig. 3), 70% of the tumors responded by regressing to less than half their original volume. However, the extent of regression was not as great as during the first course of treatment. The new tumors regressed rapidly during the first week of treatment, but thereafter little further regression occurred. Although the old tumors did not begin to respond to treatment until

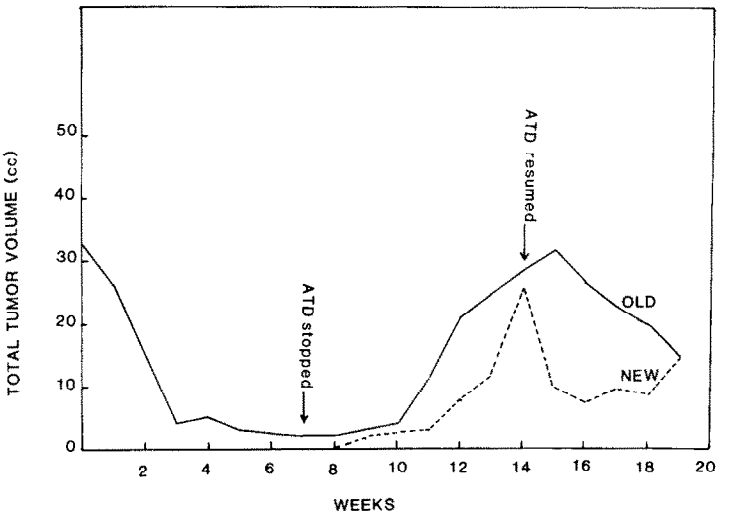

Fig. 3. Effect of two courses of ATD treatment on DMBA-induced, mammary tumors. Eight rats with twenty-eight tumors were administered ATD from subcutaneous silastic implants (150 mg) and twice daily injections (25 mg · kg^{-1} · day^{-1}). Total volume (cc) is shown of all twenty-eight tumors of treated but not control animals. Details of tumor response are shown in Table 1. Old indicates original tumors; new indicates new tumors.

Table 1. Effect of ATD treatment on DMBA-induced, hormone-dependent, mammary tumors of the rat*

			Tumor response											
			Growth		Regression									
			>50%		<50%		>50%		To <0.02 cc†		Complete		Total	
	No. of rats	No. of tumors	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Control	7	21	10	48	4	19	3	14	1	5	3	14	7	33
ATD-treated	8	29	1	3	0	0	4	14	7	24	17	59	28	97

* Rats were treated with ATD administered in silastic implant (150 mg) and by twice daily s.c. injections of 12.5 mg/kg for 7 weeks. Control animals received implants of silastic only and injections of Steroid Suspending Vehicle : Klucel (5 : 1). Implants were replaced weekly. Change in total tumor volume is shown graphically in Fig. 3.

† Tumors were too small to measure but still palpable.

Table 2. Peripheral gonadotropin and prolactin levels in rats with DMBA-induced mammary tumors after 4 weeks of treatment with ATD*

	LH (ng/ml)	FSH (ng/ml)	Prolactin (ng/ml)
Control	24.8 ± 2.9	183.4 ± 9.5	65.6 ± 12.5
ATD-treated	18.4 ± 2.2	172.3 ± 6.0	44.8 ± 9.8

* Seven rats were administered ATD from subcutaneous silastic wafers (150 mg) and twice daily s.c. injections of 12.5 mg/kg; eight control animals received implants of silastic only and vehicle injections. After 4 weeks, blood samples were collected from the carotid artery at 11 : 00 a.m. Samples from controls were collected during diestrus at 11:00 a.m. Values are means ± S.E.M. There was no significant difference ($P > 0.1$) between hormone concentrations in plasma from control and treated rats.

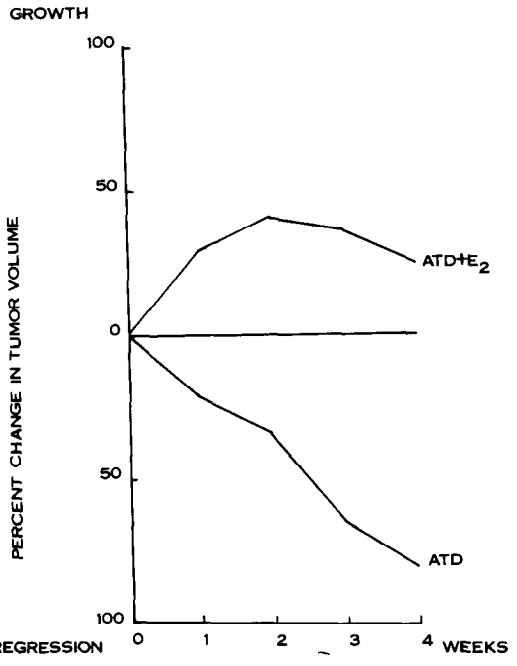

Fig. 4. Effect of adding estradiol ($0.3\ \mu g \cdot kg^{-1} \cdot day^{-1}$ injected s.c.) to ATD treatment on DMBA-induced mammary tumors. Percentage change in total volume of all tumors is shown. Two groups of six rats each were administered ATD from silastic implant (150 mg) and twice daily s.c. injections ($25\ mg \cdot kg^{-1} \cdot day^{-1}$).

the second week, most regressed to a very small size. After 5 weeks, only two rats had measurable tumors remaining. These animals were ovariectomized and adrenalectomized. None of these five tumors had responded to ATD treatment, and no further regression occurred 4 weeks after surgery.

During the 19-week experiment, the body weight changes of ATD-treated animals were within the normal range.

Studies to investigate whether other activities contribute to tumor regression

Effect of ATD on gonadotropins. In peripheral blood samples collected after the fourth week of treatment (experiment 1), gonadotropin levels were not affected by ATD (Table 2). Prolactin levels were only slightly, but not significantly, reduced in ATD-treated rats compared to controls. Control samples were drawn during diestrus since treated animals did not cycle and were in constant diestrus judged by their vaginal smears. Thus, control values represent basal levels.

Table 4. Effects of ATD and testosterone on uterine estrogen-receptor translocation*

Treatment		Translocation (Percentage of bound estrogen) Cytosol	Nuclear pellet
Vehicle		82	18
10^{-10} M	Estradiol	26	74
10^{-8} M		5	95
10^{-6} M		9	91
10^{-9} M	ATD	75	25
10^{-7} M		68	32
10^{-5} M		67	33
10^{-9} M	Testosterone	65	35
10^{-7} M		77	23
10^{-6} M		39	61

* Uteri from immature rats were incubated with [^{3}H]estradiol and one of the above compounds for 1 hr at 37° under 95% O_2:5% CO_2. After incubation, nuclear and cytosolic fractions were prepared, and estrogen receptor sites were estimated.

To further investigate whether the compound directly affected gonadotropins, ovariectomized rats were injected with ATD from the day of surgery (experiment 3). LH and FSH levels in control animals were increased markedly 8 days after surgery, as expected (Table 3). However, in animals treated with ATD from the day of surgery, there was no large increase in levels of LH. We also compared the effect of testosterone and estradiol treatment on gonadotropin levels in ovariectomized rats. No increase in LH was observed with testosterone; instead, values were significantly below the basal level of intact animals (compare Table 2). In estradiol-treated animals, LH and FSH increased to an extent similar to the controls. However, prolactin levels were increased markedly above control levels by estradiol treatment.

Competition by ATD for steroid binding sites. Receptors for progesterone, androgens, prolactin,

Table 3. Effect of ATD in ovariectomized rats*

	% Change tumor vol.†	Uterine wt (mg)	LH (ng/ml)	FSH (ng/ml)	Prolactin (ng/ml)
OVX	−55	170 ± 8^a	135 ± 5^b	905 ± 33^c	64 ± 13^c
OVX + ATD	−41	179 ± 8	53 ± 8^b	901 ± 139	55 ± 14
OVX + T	+35	581 ± 28^a	10 ± 0.7^b	377 ± 4^c	87 ± 7
OVX + E_2	+35	368 ± 16^a	130 ± 44	1292 ± 364	318 ± 83^c

* Normal rats were ovariectomized and treated from the day of surgery with one of the following regimens: vehicle, ATD (silastic implant and twice daily injections of $12.5\ mg \cdot kg^{-1}$), testosterone ($20\ mg \cdot kg^{-1} \cdot day^{-1}$) or estradiol ($0.3\ \mu g \cdot kg^{-1} \cdot day^{-1}$). Blood samples were collected on day 8 after ovariectomy. Values with the same superscripts are significantly different ($P < 0.01$) from controls (mean ± S.E.M., n = 8).

† Percent change in total volume of tumors of five rats after 4 weeks of treatment administered as above.

and glucocorticoids, as well as for estrogens, have been identified in mammary tumor tissue [24]. Androgens in pharmacological doses are known to interact with estrogen receptors [21].

To determine whether the above regression of mammary tumors resulted from additional activities of this aromatase inhibitor *in vivo*, competition of ATD for steroid binding sites was investigated. However, ATD in the range of 10^{-5}–10^{-7}M did not compete for androgen receptors. Results in Table 4 suggest that ATD at the concentrations tested had little effect in translocation of the estrogen receptor in the immature rat uterus, whereas testosterone, at similar concentrations, depleted the receptor from the cytosol and translocated it to the nucleus. Consistent with this finding, no uterotrophic effect or any significant effect on the course of tumor regression in ovariectomized rats was observed during ATD treatment. Thus, the percentage decrease in total tumor volume after 4 weeks in ATD-treated rats was similar to that in vehicle-treated ovariectomized rats (Table 3). However, when ovariectomized rats were treated with testosterone, tumors showed variable patterns of growth and regression, but by 4 weeks of treatment they had increased in total tumor volume (Table 3). The growth pattern and change in total tumor volume were similar to that seen with testosterone in intact rats.

DISCUSSION

Studies with ATD, demonstrating its properties as a competitive inhibitor of placental and ovarian aromatase, were reported previously by our group [11]. In the absence of natural substrate (e.g. androstenedione) but in the presence of NADPH, ATD also causes time-dependent loss of aromatase activity in rat ovarian microsomes. Covey and Hood [22] recently reported time-dependent loss of placental aromatase by ATD. The loss of activity appears to be more rapid in the placental than in the ovarian tissue. Despite charcoal treatment and washing of the microsomes, activity could not be restored after 24 hr.

Aromatase activity was also reduced markedly in microsomes prepared from rats injected with ATD at least 24 hr previously. Although further studies are in progress to elucidate the mechanism(s) involved in this inhibition, loss of enzyme activity would explain the lasting effects of *in vivo* treatment. Thus, both the enzyme and ovarian estrogen secretion remained inhibited for at least 24 hr after injection. A direct cause-and-effect relationship is suggested by the similarity of the patterns of aromatase activity and estrogen secretion (Fig. 2).

Following a 7-week course of ATD treatment, regressed tumors remained quiescent for 3 weeks. The mammary tumors which later grew back appeared to be growing under the influence of estrogen, as they again responded by rapidly regressing during a second course of ATD treatment. Although we have previously reported 85% reduction in estradiol secretion on proestrus in normal cycling rats treated with ATD [12], in the present study we observed no significant reduction in ovarian estrogen secretion below the basal level of controls during diestrus. The rats treated with ATD had anestrus smears and were presumably not ovulating, consistent with inhibited estrogen production during proestrus [12]. Also, when estradiol was administered with ATD, tumor regression did not occur. These observations suggest that estrogen secretion was probably reduced by ATD treatment to levels insufficient to support tumor growth.

The findings that gonadotropin levels were basal in intact rats with mammary tumors treated with ATD (Table 2) and the absence of a rise in LH following ovariectomy (Table 3) suggest an effect of gonadotropin secretion. Longer treatment, as in the tumor-bearing rats, may also affect FSH levels. Suppression of gonadotropins by ATD may be advantageous since LH and FSH have been demonstrated to be involved in control of estrogen biosynthesis [23]. Although we have not determined to what extent estrogen production may be reduced by an effect of ATD on gonadotropins, our data showing marked reduction in ovarian estradiol secretion in the PMSG-primed rats, and also previous data in normal rats [12] on proestrus, suggest action at the ovarian level.

ATD did not interact significantly with estrogen or androgen receptors and did not interfere with tumor regression induced by ovariectomy. Also, the compound did not exhibit a uterotrophic or estrogenic response (Table 3).

Our findings that testosterone stimulated mammary tumor growth in ovariectomized rats (Table 3) may be the result of interaction of testosterone with the estrogen receptor. Action of testosterone through the estrogen receptor was suggested by Zava and McGuire [21] to explain the observed growth of MCF-7 human breast cancer cells with androgens [24]. Recently, Longcope *et al.* (unpublished observations) showed that the increase in rat uterine weight by testosterone was due to estrogen receptor interaction rather than to peripheral aromatization of testosterone to estrogens.

Our studies with ATD indicate that the major *in vivo* activities of the compound are inhibition of estrogen biosynthesis and gonadotropin secretion. Both activities could contribute to regression of hormone-dependent mammary tumors and may account for the high efficacy of ATD in these studies.

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