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THE EFFECTS OF *IN VIVO* ADMINISTRATION OF 10-PROPARGYLESTR-4-ENE-3,17-DIONE ON RAT OVARIAN AROMATASE AND ESTROGEN LEVELS

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We have previously demonstrated that 10-propargylestr-4-ene-3,17-dione (PED) functioned as an irreversible inhibitor of rat ovarian aromatase *in vitro*. These studies were undertaken to examine the *in vivo* effects of PED on rat ovarian aromatase activity and estrogen production. In the current experiments, a single injection of PED (0.5 or 2.5 mg/kg) was found to maximally inhibit aromatase at 3 h regardless of dose. Significant inhibition of enzyme activity by PED was observed beyond 18 h, although some recovery was noted at the lower dose (0.5 mg/kg). Concomitantly, ovarian estrogen levels were also maximally reduced at 3 h, however ovarian estrogen levels returned toward control values prior to the recovery in enzyme activity. Even though significant inhibition of enzyme activity was observed at 12 h following a single injection of PED, the effect of double injections of the inhibitor at 12 h intervals was surprisingly not cumulative. Similarly, continued multiple injections of PED revealed significant inhibition of enzyme activity and estrogen production several hours after the injection, but variations in effectiveness were observed by 12 h which changed in accordance with a circannual cycle in aromatase. Apparently other factors are involved with maintaining aromatase levels and compensating for reduced enzyme activity. These mechanisms are evidenced by a continuation of the rat reproductive cycle with prolonged PED administration and a reduced influence of PED in regard to enzyme inhibition at certain times of the year. Despite these variations in the duration of action of PED, no comparable changes were observed in effectiveness as an anti-tumor agent. These results suggest that complex mechanisms exist which regulate the activity of aromatase in order to maintain estrogen production. Further research using compounds such as PED may assist in elucidating the factors that modulate ovarian estrogen production.

KEY WORDS: Aromatase, aromatase inhibitors, estrous cycle, ovarian steroid levels.

INTRODUCTION

Aromatase, the enzyme that converts androgens to estrogens, has been the subject of intensive study as a control point for estrogen biosynthesis.[1,2] It is also a target enzyme for the treatment of hormone-dependent disorders.[3,4]

Previous studies in this laboratory established that the rat ovarian aromatase is similar in most respects to that from human placenta.[5] Using the rat as a model,

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10-propargylestr-4-ene-3,17-dione (PED) was found to be an effective mechanism-based irreversible inhibitor of rat ovarian aromatase *in vitro*.[5,6] In view of the potency of PED in the *in vitro* assays, we subsequently examined PED as a potential therapeutic agent for hormone-dependent cancer using the dimethylbenzanthracene-induced rat mammary tumor model. PED treatment at both a high dose (50 mg PED/kg body weight/day) and a low dose (1 mg/kg/day) resulted in either regression or cessation of hormone-dependent tumor growth irrespective of dose.[7,8]

In the course of the mammary tumor studies it was observed that submaximal doses of PED resulted in marked effects on tumor growth, while the animals apparently maintained their estrous cycles. This was a surprising observation, since both the growth of the tumors and the events of the estrous cycle are thought to require the action of estrogens.

The current study was therefore designed to examine the effects of PED on aromatase and estrogen levels *in vivo*. Given the fact that rats have a four day estrous cycle, rapid changes in enzyme and hormone levels must occur, and it was therefore necessary to examine the duration of the effect of PED. The effects of a single administration of PED were then compared to the effects of long-term treatment.

MATERIALS AND METHODS

Supplies

Trizma base, bovine serum albumin, gelatin, NADPH, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Company, (St. Louis, MO). Norit A charcoal and sodium and potassium salts were purchased from Fisher Scientific Company, (Pittsburgh, PA). Dextran T-70 was obtained from Pharmacia Fine Chemicals, (Uppsala, Sweden). [1,2,6,7-^{3}H(N)] Androstenedione, [4-^{14}C] androstenedione, [1,2,6,7-^{3}H(N)] testosterone, [2,4,6,7-^{3}H(N)] estradiol, [2,4,6,7-^{3}H(N)] estrone, [1,2,6,7-^{3}H] progesterone, [1β, 2β-^{3}H(N)] testosterone, and [1β-^{3}H(N)] androstenedione were purchased from New England Nuclear (Boston, MA), PED was synthesized as described[9]; all other steroids were acquired from Steraloids, (Wilton, NH). The estradiol antiserum was a gift of Dr. G. Niswender, while the estrone antiserum was purchased from Radioassay Systems Laboratories, Inc. (Carson, CA). Dioxane, xylene, naphthalene, and 2,5-diphenyloxazole were obtained from Research Products International Corp. (Elk Grove Village, IL). Aqueous counting scintillant was acquired from Amersham Corp. (Arlington Heights, IL). Virgin CD females (55 days old) were procured from Charles River Breeding Laboratories, Inc. (Wilmington, MA).

Animal Protocol and Assays

All animals were maintained at 22–24°C on a 12:12 light:dark cycle. The rats were allowed 4–8 days of acclimatization to the Animal Care Facility. Following this period, the stage of the estrous cycle was assessed by daily vaginal smears for at least two weeks. Only those animals exhibiting at least two consecutive regular cycles were used in the studies. Entry into the relevant cycle stage was verified on the day of sacrifice.

The animals were injected subcutaneously with 0.1 ml of either sesame oil (controls)

or PED in sesame oil (corresponding to about 0.5 and 2.5 mg PED/kg body weight). In the proestrus time-course experiments injections were performed at zero time, i.e. 30–45 min after the initiation of the light period in the animal facility, and the animals sacrificed at various times thereafter. In the long-term studies the injections were performed 12 h apart; unless otherwise stated the animals were sacrificed 12 h after the last injection.

Groups of 3–4 rats were sacrificed by carbon dioxide asphyxiation, followed by exsanguination via the vena cava. The ovaries were removed, dissected free of adhering tissue, and frozen on dry ice. Microsomes were prepared by differential centrifugation and assayed for aromatase activity by a tritiated water release method as previously described.[5,10,11] Estradiol and estrone levels were determined in the ovarian cytosol from the same ovaries by specific radioimmunoassay using standard procedures.[12]

All values are expressed as mean ± SEM. All times are given in hours after the beginning of the light period in the animal facility. Statistical significance was determined by Student's t test.

RESULTS

Proestrus PED Time-course Experiment

Since rat ovarian aromatase activity is highest on the day of proestrus, this day was chosen to examine the effects of a single injection of PED. Animals were injected on the morning of proestrus with either PED (0.5 and 2.5 mg/kg) or sesame oil and sacrificed at various intervals.

The PED-treated animals exhibited significantly lower aromatase activity than the controls for up to 18 h after the injection (Figure 1A). At both doses of PED, maximal inhibition was observed at 3 hours (60 ± 6% and 75 ± 4% for the 0.5 and 2.5 mg/kg doses of PED, respectively). At the lower dose of PED, a partial recovery toward control activity was observed; however, full recovery was not observed until 24 h after the treatment (data not shown). Interestingly, the predominant effect of increasing the concentration of PED was to lengthen the time of recovery toward control levels; the degree of inhibition at the 3 h time-point was surprisingly similar. It is evident that PED affected estradiol levels; there was a 40% decrease in ovarian cytosolic estradiol at the 3 h time point regardless of dose (Figure 1B). Unexpectedly, the estradiol levels recovered to nearly control levels by the 9 h time point, even in the face of continued inhibition of aromatase activity (65% inhibition for the higher dose of PED). Likewise, the estrone levels responded to PED to the same degree as estradiol and showed similar recovery kinetics (data not shown). In all experiments PED exhibited significantly greater inhibition of ovarian aromatase activity than of estrogen levels. The return of estrogen levels toward control values prior to the recovery in enzyme activity suggests a dissociation between estrogen production and enzyme activity.

PED Double-injection Experiment

The PED time-course experiments indicated that the effect of the inhibitor on ovarian aromatase activity persisted for at least 12 h following treatment. Since the enzymatic activity normally decreases following the proestrus peak, it was expected that re-

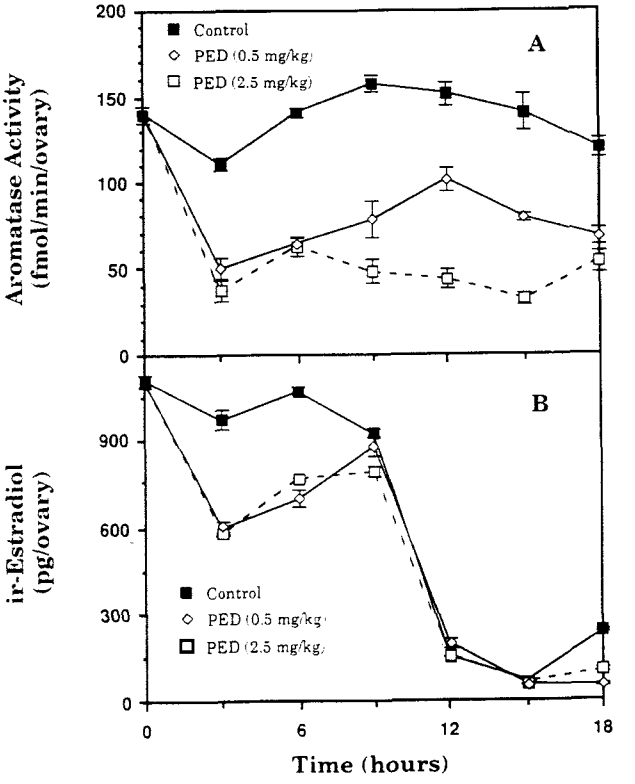

FIGURE 1 Effects of PED on Ovarian Aromatase Activity and Estradiol Levels. Groups of rats were injected with either 0.5 mg/kg or 2.5 mg/kg PED or injection vehicle on the morning of proestrus. Rats were sacrificed at timed intervals, ovaries quickly excised and frozen for subsequent analyses. **Panel A**. Ovarian aromatase activity is expressed as fmol estrogen produced per min per microsomal fraction normalized to a single ovary. The activity in PED-treated animals was significantly less than controls at all timepoints (at least $p < 0.01$ for 0.5 mg/kg PED and $p < 0.001$ for 2.5 mg/kg). **Panel B**. Immunoreactive ovarian estradiol levels are expressed as pg estradiol/ovary. All samples were extracted on Sep-Pak C-18 columns prior to assay. Significant differences between control and PED-injected animals were observed at 3 and 6 h ($p < 0.001$). At 9 h, significant differences were seen for the higher dose ($p < 0.001$), but no differences were evident after that time.

peated injection of an irreversible inhibitor such as PED at 12 h intervals would lower the residual activity essentially to zero. On the other hand, animals receiving as much as 10 mg PED/kg/day were found to continue ovulatory estrous cycles (data not shown). In order to examine this phenomenon the following experiments were carried out.

On the morning of proestrus animals were injected with sesame oil or 0.5 mg/kg PED. Some animals were sacrified at 3 and 12 h. At the 12 h time point the PED-injected animals received either sesame oil or a second injection of PED. Likewise, the vehicle-injected controls received PED (0.5 mg/kg) or a second injection of oil. The four separate groups were then sacrificed 12 h later.

At 3 and 12 h after the first injection of PED, a 40% inhibition of aromatase activity was noted (Figure 2). As had been observed in the proestrus time-course experiments,

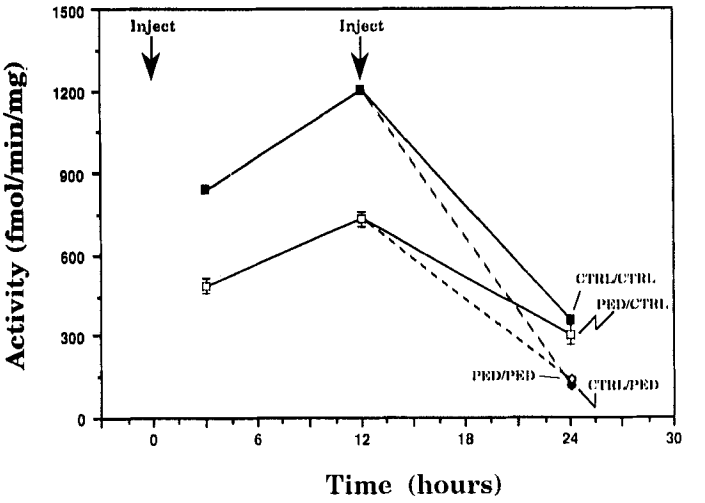

FIGURE 2 Effects of Two Injections of PED on Ovarian Aromatase Activity. Adult female rats were injected with either sesame oil or 0.5 mg/kg PED on the morning of proestrus. Some animals were killed at 3 and 12 h. At 12 h, PED-injected animals received either a second injection of the compound or the injection vehicle. Similarly, control animals received a second injection of oil or an injection of PED. Aromatase activity is reported as fmol estrogen/min/mg microsomal protein. Significant inhibiton was observed 3 and 12 h after a PED injection ($p < 0.001$). Administration of PED at 12 h to either controls or PED-injected animals resulted in a similar degree of inhibition at 24 h ($p < 0.001$), however, no cumulative effect of PED was observed.

by 24 h the animals receiving PED only at zero-time had activities comparable to control levels. The interesting observation in this experiment is that the groups of animals receiving PED at the 12 h time point had similar activities at 24 h, irrespective of the character of the first injection. Therefore, the effect of PED does not appear to be cumulative, since two injections result in the same level of activity observed following a single injetion. Although clearance of PED may be involved with this phenomenon, the fact that the aromatase activity in these two groups differed by nearly a factor of two at the time of the second injection suggests that there are compensatory mechanisms that regulate ovarian aromatase activity.

PED Multiple Injection Experiment

The double-injection experiment suggested that the effects of two injections of PED were not additive under the conditions used. More prolonged treatment was therefore assessed for effect on the ovarian aromatase activity.

Animals were injected with the sesame oil carrier or with two submaximal doses of PED (1 or 5 mg/kg/day); all the animals were sacrificed after two weeks of treatment, 12 h after the final injection. Significant inhibition of aromatase was observed at both doses on each day of the cycle (Figure 3), but no dramatic dose-dependence was observed under these condition. In fact, equivalent doses of PED appeared to have varying efficacies on each day of the cycle. For example, on proestrus, 5 mg/kg PED daily inhibited enzyme activity more than 50% (825 ± 9.9 *vs* 374.9 ± 25.3, $p < 0.001$), while on diestrus, enzyme inhibition was considerably less (413 ± 22.2 *vs* 293.7 ± 18.2, $p < 0.02$).

In this multiple injection experiment the ovarian estradiol levels were inhibited on

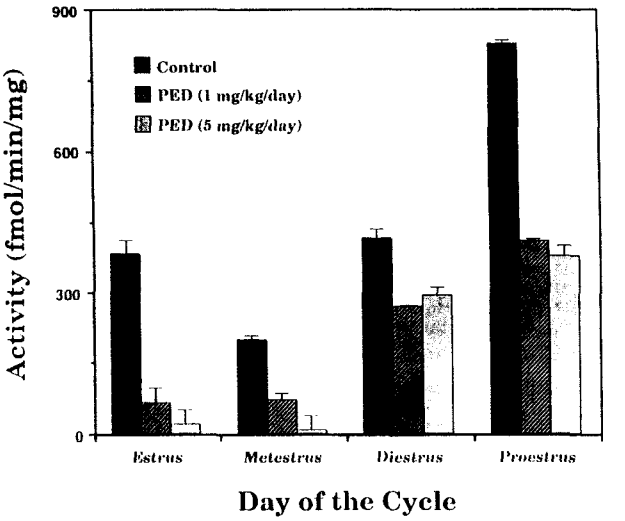

FIGURE 3 Effects of Repeated Injection of PED on Ovarian Aromatase Activity. Groups of rats were sacrificed following two weeks of twice daily injections of either 1 mg/kg, 5 mg/kg PED or oil. All PED-treated groups displayed significantly less aromatase activity relative to controls. But as seen in the double injection experiments no cumulative effects of PED were observed.

the day of proestrus. Twelve hours after the last injection, as with the time-course experiments (Figure 1), the magnitude of inhibition of the estradiol levels (24.6 ± 2.3% for 1 mg/kg/day and 41.7 ± 3.8% for 5 mg/kg/day) was again less than that of aromatase (50.5 ± 1.1% and 54.6 ± 3.1%, respectively). In contrast, the effect of PED on estradiol levels was more marked three hours after the last injection on proestrus; inhibition of estradiol production (53.6 ± 0.9% for 1 mg/kg/day and 81.0 ± 0.7% for 5 mg/kg/day) was comparable to that observed for aromatase activity (47.7 ± 1.7% and 83.3 ± 2.2%, respectively).

Over the course of our PED-injection experiments we have observed a seasonal variation in baseline ovarian aromatase activity in the rat.[13] Despite this seasonal variation in enzyme activity, no changes were observed in peak estrogen production on the day of proestrus (data not shown). This dissociation between enzyme and product was not due to limiting substrate levels or the affinity of the enzyme for its substrate. Peak androgen levels were sufficient to saturate at least 90% of the enzyme throughout the year. However, correlated with this circannual variation we have observed in long term experiments a varying *in vivo* effectiveness of PED on rat ovarian aromatase. Animals chronically treated with PED in winter do not show any significant enzyme inhibition by 12 h after the injection (Figure 4). In contrast, animals treated with PED in summer, when baseline enzyme activity is higher, do display inhibited enzyme activity at 12 h, but the residual activity is comparable to that seen in the winter in both treated and untreated animals. This was observed throughout the estrous cycle.

Regardless of the time of year, we have previously observed that submaximal doses of PED caused tumor regression without coincident cessation of the rat reproductive cycle.[8] To investigate the effects of PED on reproductive cycles more closely, we performed daily vaginal smears on rats given 1 or 5 mg/kg/day PED. Prior to the initiation of these experiments approximately 90% of the rats displayed regular 4 day

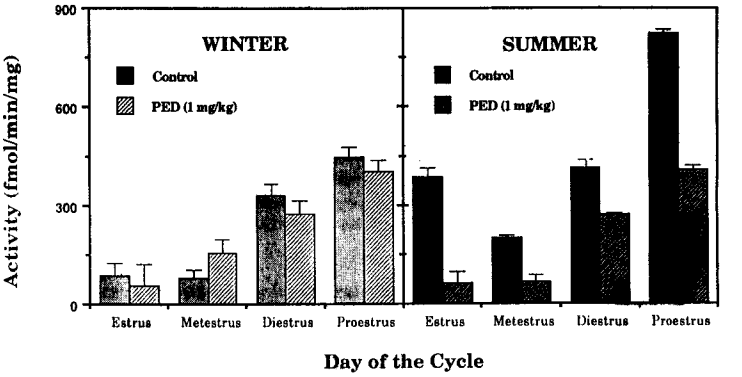

FIGURE 4 Effects of PED on Aromatase Acitivity during the Year. Groups of rats were sacrificed on the morning of each day of the estrous cycle following at least a week of PED administration (1 mg/kg/day) or control oil. In the winter, no differences occurred between treated and control animals, while in the summer, the PED-treated animals exhibited between 35% (diestrus) to 83% (estrus) inhibition of enzyme activity relative to controls ($p < 0.005$).

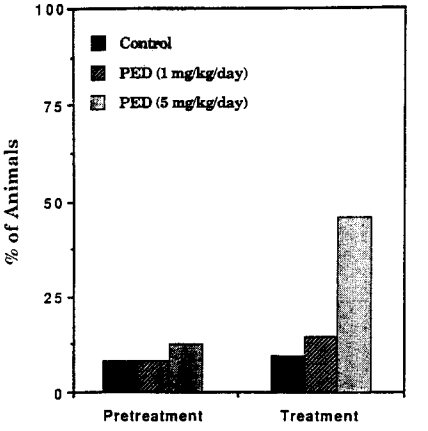

FIGURE 5 Alteration in Estrous Cycle Length by PED. Adult female rats were maintained on a 12:12 light: dark cycle and vaginal smears taken daily to monitor the estrous cycle. Prior to initiation of treatment approximately 90% of all rats had regular 4 day cycles. Rats were randomly assigned to a control group or two PED groups (either 1 mg/kg/day or 5 mg/kg/day) After at least two weeks of treatment PED-treated rats displayed a dose-dependent shift to 5 day cycles (50% at the higher dose).

cycles. No alterations in estrous cycle were ever observed in the control animals. In contrast, animals receiving either 1 or 5 mg/kg of PED daily showed a dose-dependent lengthening of the cycle to 5 days (Figure 5). In fact, at 5 mg/kg more than 50% of the rats had five day cycles. No further lengthening or irregularities were seen in any group.

DISCUSSION

A single injection of PED results in a potent inhibition of ovarian aromatase activity and estrogen levels. However, estrogen production returns to control levels prior to

any significant recovery in enzyme activity. Furthermore, even though a single injection of PED inhibited aromatase activity for more than 12 h, injection of PED at 12 h intervals was not cumulative. In fact, animals injected with PED for a prolonged period of time have an apparently normal estrous cycle, although there is a dose-dependent shift toward a 5 day cycle. Moreover, we have observed a circannual cycle in aromatase activity and a coincident variation in the duration of the effects of PED on enzyme activity. However, neither the effects of PED on reproductive cycles nor the efficacy of PED as an anticancer agent are affected by the time of year. Since PED does not bind steroid receptors and has minimal biological activity,[6,14] the observed transient lowering of estrogens, induced by PED, is apparently sufficient to produce these effects on tumor regression and on the shift to 5 day cycles.

Previous studies in this laboratory suggested that at proestrus the ovarian androgen levels are sufficient to saturate the aromatase enzyme.[15] Assuming that the enzyme is limiting, one would predict that inhibition of aromatase activity by PED would result in a substantial reduction in estrogen production. Instead, while a single injection of PED had a significant effect, it was invariably to a lesser degree than the effect on the enzyme levels, and in some experiments, the estrogen production returned to control levels in the face of continued inhibition of aromatase. Thus, dissociations between estrogen production and aromatase activity had been observed both in the presence (e.g. Figure 1) and absence of inhibitors,[15] again suggesting that not all the aromatase enzyme is totally active. In our previous studies we have seen a divergence of ovarian aromatase activity from estrogen production in normal adult cycling rats.[15] Over the course of our experiments with PED we observed that ovarian enzyme activity changes in a circannual pattern without concomitant changes in proestrous peak estrogens. It is therefore likely that some of the enzyme is not involved with estrogen production at certain times of the year. In addition to our results in the cycling rat, Hickey *et al.* have reported a dissociation between ovarian enzyme levels and estrogen production during rat pregnancy.[16]

The fact that the amount of estrogen produced does not vary proportionately with the aromatase activity therefore suggests that the *in vitro* measurement of ovarian aromatase activity is in some degree misleading. One possible mechanism is that some fraction of the aromatase cytochrome P-450 is not associated with the cytochrome reductase *in vivo* (i.e. existing as latent enzyme), but that the microsome preparation procedure destroys the functional separation between the two proteins. It is possible that when the estrogen production decreases as a result of an inactivation of physiologically active enzyme, some of the latent enzyme is activated. Payne and Valladares have observed an acute activation of aromatase in the testis in response to gonadotropin stimulation;[17] whether similar mechanisms exist in the ovary is currently unknown. However, the increase in estrogen production without alteration in the apparent aromatase activity would support such a mechanism (see Figure 1). Upon perturbation with PED *in vivo*, the latent aromatase which is not vulnerable to inactivation by PED, may become associated with reductase, thereby activating the enzyme. Repeated administration of PED may reduce the pool of latent enzyme resulting in the greater effects of PED on estradiol levels seen in the multiple injection experiments. Of course, with chronic administration other factors, including increased enzyme synthesis may also play a role. Although the submaximal doses of PED used in these experiments, may not be able to overcome the compensatory mechanisms of the ovary, higher doses of PED (50 mg/kg/day) were found to stop the estrous cycle and inhibit aromatase activity by greater than 90% at 12 h.[8]

Another explanation for this phenomenon is that some of the androgen may be sequestered from the ovarian enzyme such that the available substrate may be limiting for estrogen synthesis.[18,19] This sequestration appears less likely than the possibility of latent enzyme because of the ready diffusion of steroids throughout most tissues. Alternatively, it is possible that an endogenous inhibitor is present in the ovary and dissociates from the enzyme upon homogenization. Several groups have reported evidence for the existence of such an inhibitor,[20,21] but very little is known about its biochemical nature or its role in modulating aromatase activity.[22,23]

In contrast to the apparently altered response of the ovarian aromatase activity to PED at different times during the year, administration of PED resulted in tumor regression at the same rate independent of circannual effects.[8] The tumor regression is probably a result of estrogen-mediated effects, since PED did not have any apparent deleterious effects on the animals and does not have any significant biological effects other than inhibiton of aromatase.[6,14] Since the rats maintained their estrous cycles relatively unimpaired, this suggests that the tumor growth is regulated in a manner qualitatively distinct from that of the cycle. Whether tumor growth is more sensitive to relatively transient decreases in estrogen production, or perhaps to other, as yet uncharacterized, factors common to aromatase inhibitors remains to be addressed experimentally.

In summary, PED is a potent inhibitor of rat ovarian aromatase *in vivo*, and may prove useful as a treatment for endocrine-related disorders. In addition, the use of aromatase inhibitors such as PED may assist in shedding light on the complex regulatory mechanisms involved in controlling ovarian estrogen production.

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