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Report

Effect of CGS 20267 on ovarian aromatase and gonadotropin levels in the rat

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Summary

Aromatase catalyzes the rate limiting step that converts androgens to estrogens. Postmenopausal women with hormone dependent breast cancer respond to first generation aromatase inhibitors such as aminoglutethimide with a marked suppression of circulating estradiol levels. In contrast, premenopausal women appear to be resistant to first generation aromatase inhibitors. The inability to block ovarian aromatase results from the low affinity of first generation inhibitors for the active site of the enzyme. Under these circumstances, the high substrate levels in the premenopausal ovary compete effectively with these inhibitors and do not allow binding of inhibitor to the active site of the enzyme.

Second and third generation aromatase inhibitors with higher affinity for aromatase have now been developed and potentially could block ovarian aromatase. To test this possibility, we administered CGS 20267 (letrozole), a highly potent aromatase inhibitor, to cycling female rats. A dose dependent inhibition of uterine weight occurred with maximum effects produced by the 5 mg/kg/day dosage. During a period of 4 weeks, uterine weight was reduced to levels induced by ovariectomy. Ovarian tissue estradiol levels were inhibited by approximately 80%. As a reflection of inhibition of ovarian aromatase activity, the levels of androstenedione in the ovary increased by an order of magnitude. Both LH and FSH plasma levels increased but not to those observed after ovariectomy. The rise in gonadotropin levels induced a statistically significant but relatively small increase in ovarian weights.

These results demonstrate the ability to persistently block ovarian aromatase activity in cycling rats with a potent aromatase inhibitor. This study provides a rationale for clinical trials of potent aromatase inhibitors in pre-menopausal women with breast cancer.

Introduction

One-third of human breast cancers depend upon estrogen for growth and regress with inhibition of estrogen biosynthesis or blockade of its action [1]. The rate limiting step in estrogen synthesis is the conversion of androgens to estrogens through the enzyme aromatase. Present in ovary, fat, muscle, liv-

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er, and in breast cancer tissue itself, aromatase specifically catalyzes the conversion of androstenedione to estrone and testosterone to estradiol [2].

First generation aromatase inhibitors, such as aminoglutethimide, effectively inhibit estrogen synthesis in postmenopausal women and cause regression of breast cancer lesions [3]. Aromatase exists primarily in extra-gonadal sites in postmenopausal women [2]. The levels of the aromatase substrates, androstenedione and testosterone, are relatively low in these peripheral tissues. For this reason, competitive inhibitors with low affinity for the active site of the enzyme can effectively compete with substrate and block aromatization. In marked contrast, the majority of aromatase is in the ovary in premenopausal women and very high amounts of substrate are present there. For this reason, aminoglutethimide, which has a low affinity for the active site of aromatase [4], causes only partial inhibition of estradiol production in premenopausal women [5]. In addition, lowering of estradiol interrupts negative feedback and causes a reflex rise in LH and FSH levels [5]. LH stimulates the ovary to produce even more androstenedione as substrate and FSH augments the amount of aromatase present [2]. These reflex effects further interdict the ability of aminoglutethimide to block aromatase. Consequently only luteal phase estradiol levels fall significantly in response to aminoglutethimide in premenopausal women, but the reduction is not to castrate levels. These data illustrate the need for inhibitors with greater affinity for the active site of aromatase.

Over the past two decades, second and third generation aromatase inhibitors with 3–4 orders of magnitude greater potency than aminoglutethimide have been developed [4, 6]. Whereas doses of 500–1000 mg of aminoglutethimide were used daily in patients with breast cancer, doses of 0.1 mg daily of letrozole maximally suppress estradiol in postmenopausal women [7]. This greater potency results from a higher affinity of the drug for the enzyme's active site as well as a slower rate of plasma clearance. Substantially higher doses than 0.1 mg have been used and are non-toxic, and well tolerated [7, 8].

Based upon these observations in post-menopausal women, it appeared likely that high doses of letrozole might be capable of blocking aromatase in the ovary in pre-menopausal women. Accordingly, we initially sought to determine whether letrozole could block estrogen synthesis in an animal model, the cycling rat. Our findings demonstrated effective inhibition of ovarian aromatase activity even in the presence of reflex increments in LH and FSH levels.

Methods

Animal studies

Two studies were conducted: a trial of increasing letrozole doses to determine the amount required to effectively inhibit aromatase in the ovary; and a second trial to examine the time course of biochemical and hormonal effects. Both studies used 2–3 month old Sprague-Dawley rats (from Charles River for the dose reponse study and Harlan for time study). For the dose-response study, 60 rats were segregat-

Table 1. Animal body weight

Group	Study	Pretreatment	1 week	2 weeks	4 weeks
vehicle	time course	229 ± 3.6	235 ± 4.2	246 ± 3.4	260 ± 2.2
ovex	–	247 ± 5.4	252 ± 4.4	274 ± 1.4	301 ± 4.5
letrozole	–	239 ± 3.7	257 ± 3.3	273 ± 5.9	313 ± 4.8
vehicle	dose response study	320 ± 4.3			328 ± 7.1
ovex	–	328 ± 4.3			382 ± 6.2
0.05 mg/kg/day	–	316 ± 3.6			344 ± 6.0
0.5 mg/kg/day	–	335 ± 5.6			379 ± 7.0
5.0 mg/kg/day	–	319 ± 4.3			361 ± 6.2

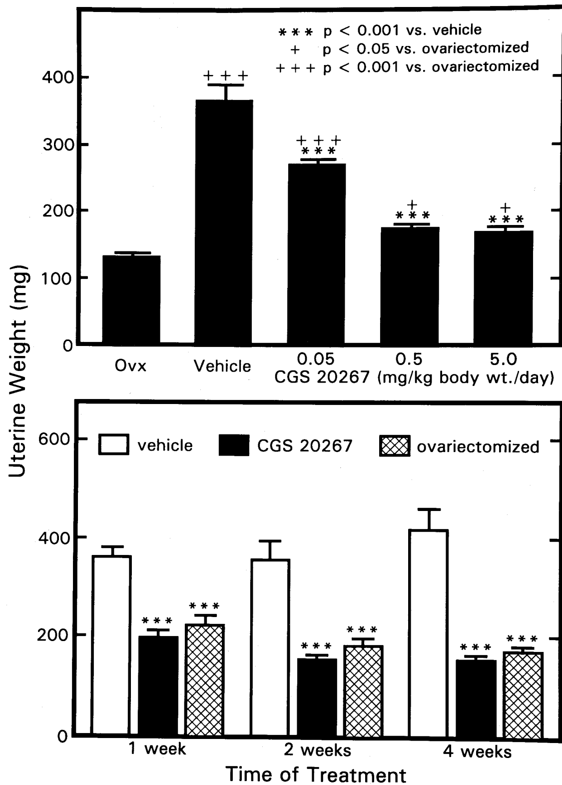

Figure 1. Uterine weights during dose response study (top panel) and time course (5 mg/kg/day) study (bottom panel). Error bars represent mean ± SEM.

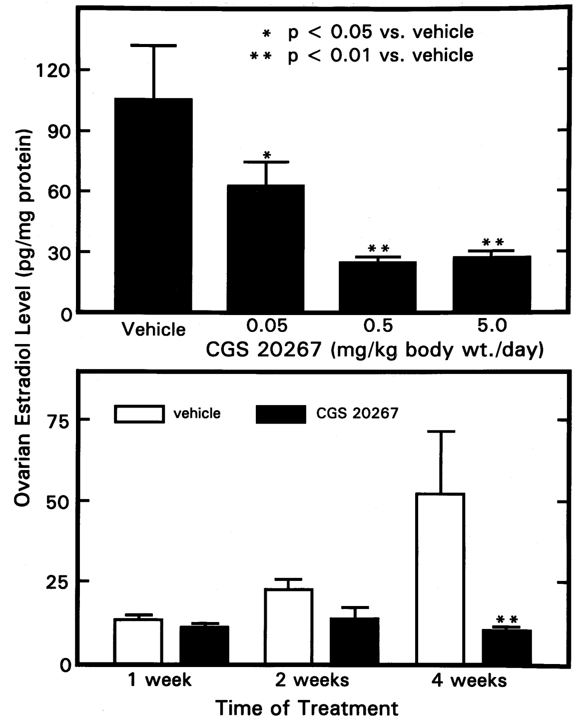

Figure 2. Ovarian estradiol levels during dose response study (top panel) and time course study (bottom panel). Error bars represent mean ± SEM.

ed into five groups; ovariectomized, (2) vehicle-treated, and (3–5) three groups that received letrozole at doses of 0.05, 0.5, or 5.0 mg/kg body weight/day. The drug was solubilized (suspended at the highest dose) in 0.5% high viscosity carboxymethylcellulose and administered by gavage for four weeks. In the time course study, 108 animals were split into three groups: ovariectomized, vehicle-treated, and letrozole-treated at 5 mg/kg body weight/day. Twelve animals in each group were sacrificed at 1, 2, and 4 weeks. All studies were carried out according to animal use guidelines established by NIH and the institutions where the studies were carried out.

Animals were weighed at the beginning and end of each study to document the lack of severe systemic toxicity (Table 1). When the animals were sacrificed, serum was obtained and stored at – 20 degree C until assay of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol. At the termination of the study, ovarian and uterine weights were measured and the ovaries frozen at – 70 degree C for later extraction and measurement of estradiol.

Hormone assays

Serum FSH and LH were measured by radioimmunoassay [9]. The ovarian androstenedione and estradiol radioimmunoassays were performed after homogenization of the samples in phosphate-buffered saline and extraction by ether [10].

Data analysis

Data were analyzed by one-way ANOVA with Newman-Keuls means testing. Due to technical difficulties with the gonadotropin radioimmunoassays, values more than 10 standard deviations from group means of the remaining samples were exclud-

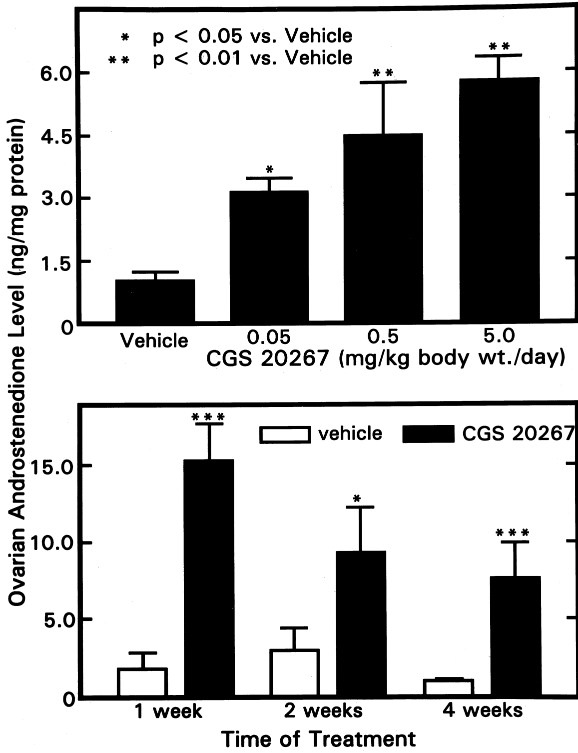

Figure 3. Ovarian androstenedione levels during dose response study (top panel) and time course study (bottom panel). Error bars represent mean ± SEM.

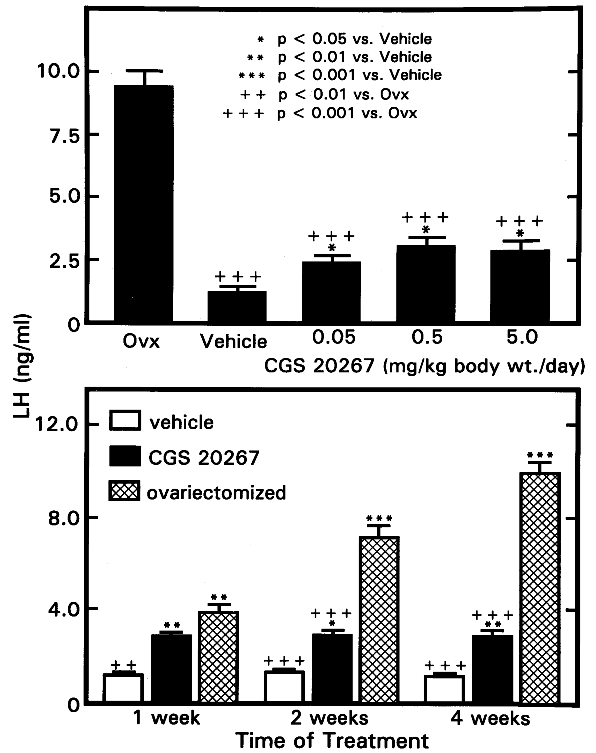

Figure 4. Plasma LH levels during dose response study (top panel) and time course study (bottom panel). Error bars represent mean ± SEM.

ed from analysis. Data from one animal in the two-week vehicle group has been deleted as it was discovered at the time of sacrifice that it had only one ovary. This anomaly was assumed to be present prior to the beginning of the study as carboxymethylcellulose has been used routinely and has never been shown to have any effects on the reproductive system.

Results

We utilized measurements of uterine weight initially as a means to bioassay the reduction in estradiol levels achieved by ovariectomy or with the administration of the aromatase inhibitor. Letrozole caused a dose dependent reduction in uterine weight with maximal effects observed at the 5 mg/kg body weight/day dose; this response approached the effects seen following ovariectomy (Figure 1 top). The time course (Figure 1 bottom) study demonstrated that letrozole and ovariectomy caused parallel decreases in uterine weight at the one, two, and four week time points.

Measurements of the ovarian estradiol levels provided a sensitive means of directly demonstrating inhibition of estrogen biosynthesis. Basal levels of 107 ± 24 pg/mg protein fell maximally to 18.9 ± 6.9 pg/mg protein in response to the 5.0 mg/kg/day dose of letrozole (Figure 2 top). During the time course study, levels at one, two, and four weeks remained in the same range without evidence of escape at the four week time point (Figure 2 bottom). Of interest were the low estradiol levels in the vehicle-treated rats and the rise over the four weeks of observation which probably reflected an effect of puberty in these young animals. This conclusion was supported by the fact that the vehicle-treated animals before the time course study weighed only 229 ± 3.6 gm whereas the vehicle-treated animals used for the dose reponse study weighed 320 ± 4.3 gm at the outset (Table 1).

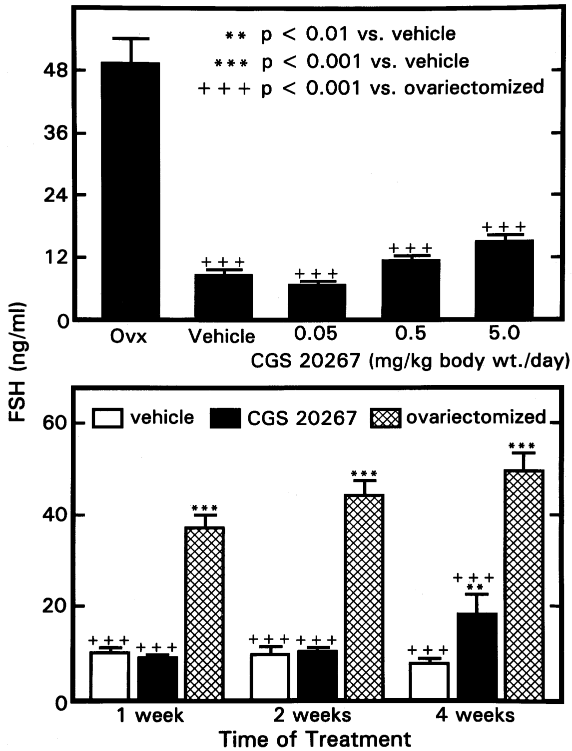

Figure 5. Plasma FSH levels during dose response study (top panel) and time course study (bottom panel). Error bars represent mean ± SEM.

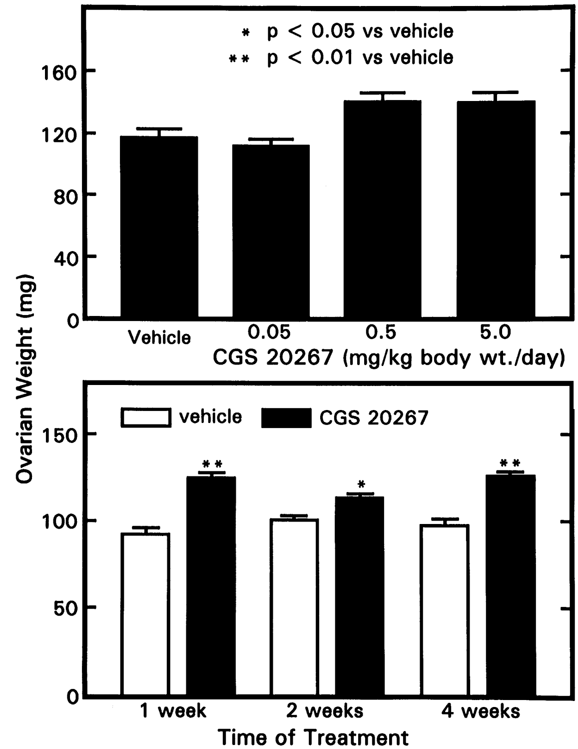

Figure 6. Ovarian weights during dose response study (top panel) and time course study (bottom panel). Error bars represent mean ± SEM.

To obtain further evidence of the effects of letrozole on the ovary, we measured ovarian androstenedione levels. During the dose reponse study ovarian androstenedione levels increased from 1.06 ± 0.23 ng per mg tissue protein in the vehicle-treated animals to 5.68 ± 0.59 ng/mg tissue protein in the animals receiving 5.0 mg/kg/day of letrozole. During the time course study, androstenedione levels peaked at 15.6 ± 3.2 ng per mg tissue protein on week one and fell slightly to 7.7 ± 2.3 ng/mg tissue protein at week four. Levels remained stable in the vehicle-treated animals.

Measurement of plasma LH and FSH levels allowed assessment of potential reflex increments in response to the inhibition of estradiol synthesis. In the dose response study, LH levels in the vehicle-treated animals were 1.1 ± 0.2 ng/ml versus 2.8 ± 0.4 ng/ml ($p < 0.001$) in the animals receiving 5.0 mg/kg/day of letrozole over a four week period. This increase was substantially less than that observed in the ovariectomized animals (9.3 ± 0.7 ng/ml). During the four week time study, the LH levels in the letrozole-treated animals remained in the range observed during the dose reponse study and were 2.8 ± 0.3, 3.0 ± 0.3 and 2.9 ± 0.3 at one, two, and four weeks (Figure 4 top and bottom). Levels in the vehicle-treated animals did not change whereas those in the ovariectomized animals increased from 3.7 ± 0.5 to 9.9 ± 0.7 over the four week period, again probably reflecting progression of the pubertal process. Patterns were quite similar with respect to FSH levels although increases in response to letrozole were less significant. The only statistically significant change in FSH levels in response to letrozole occurred at the four week point in the time course study (Figure 5 top and bottom).

Further evidence of reflex increments in LH and FSH could be obtained by measurement of ovarian weights during the administration of letrozole. A trend toward ovarian enlargement in animals receiving letrozole was observed during the dose response study but these differences in weight were

not statistically significant. During the time course study, ovarian weight was consistently and significantly increased in the letrozole-treated animals. While significant, the magnitude of difference was not great, with ovarian weights of 98 ± 4 mg in the vehicle-treated animals and 124 ± 3 mg in the letrozole treated animals at four weeks ($p < 0.01$) (Figure 6 top and bottom).

Discussion

Letrozole is a third generation aromatase inhibitor which is 3–4 orders of magnitude more potent than the first generation aromatase inhibitor, aminoglutethimide [4, 6]. At doses of 0.5 to 5.0 mg/kg/day, letrozole blocks ovarian estradiol synthesis substantially in rats as reflected by an 80% reduction in ovarian estradiol concentrations. Using uterine weight as a bioassay for systemic estrogen effects, letrozole appears to produce biologic actions similar to those induced by surgical oophorectomy. The doses used in this study would be the bioequivalent of 5 to 50 mg daily in a 70 kg woman when calculated from body surface area proportionality. Prior studies demonstrated the safety of a 5 mg daily dose in patients studied short term and 2.5 mg doses used for up to one year of administration [7–8, 11]. Thus it is possible that letrozole could effectively inhibit ovarian aromatase in premenopausel patients.

The reflex increments in LH and FSH caused an increase in ovarian weight in the animals studied. At the time of design of this study, it was not known whether gonadotropins could exert mitogenic effects on the ovary in the absence of estradiol, an hypothesis raised initially by Reiter et al. [12]. The demonstration of increased ovarian weights in this study would mitigate against a permissive role of estrogen in gonadotropin-induced mitogenesis. However, we did not achieve complete inhibition of ovarian estradiol levels and therefore cannot definitively make conclusions about this issue. More recent data from two sources suggest that estradiol is not necessary for this permissive effect. Animals with knockout of their estrogen receptor and women with aromatase deficiency have enlarged ovaries in response to gonadotropin increments [13–14]. Based upon these observations, it is likely that aromatase inhibition, even if complete, will result in ovarian enlargement in patients. Whether or not this effect will be problematic clinically is unknown at present.

Our studies attempted to measure estradiol levels in serum to further document the degree of estradiol suppression. Using a sensitive radioimmunoassay as well as a recombinant DNA yeast-based bioassay [7], we could detect no significant effect of oophorectomy on serum estradiol levels (data not shown). We believe that these observations reflect either the lack of sensitivity of estradiol assays in animals without estrogen-binding proteins or the presence of blank material or cross-reacting substances in the sera of these animals. Measurements of estradiol in the ovary itself, where levels are substantially higher than in serum, provided a means of accurately assessing estradiol production. In addition, the measurement of uterine weight provided a sensitive and precise bioassay of the estrogen effect.

A confounding problem in the performance of this study was the difference in body weight between animals in the time course and dose response studies. The age of animals at the start of the experiments differed only by one week. However, presumably because of differences introduced by different suppliers, the weights of animals differed substantially. Because the heavier group of animals was likely progressing through puberty during observation, hormone levels differed between animals studied in the two groups. While problematic, the data are readily interpretable since appropriate controls were used in each arm of the study.

This study confirms and extends the studies of Schieweck et al. [15] who have observed similar uterine weight suppression in response to letrozole. Our study and that of Schieweck et al. demonstrated no or only minimal increments in FSH. This may reflect the rise in androstenedione levels expected from the increments of ovarian androstenedione. Alternatively, the levels of inhibin may increase during letrozole administration and dampen the expected rises in FSH. Differentiation between these two possibilities will require further study.

The results reported in this and other studies, suggest the feasibility of blocking ovarian aroma-

tase in premenopausal patients. The substantially higher affinity of newer aromatase inhibitors for the aromatase enzyme should make it possible for these agents to compete successfully for the enzyme activity site. However, species differences are substantial and carefully conducted investigations in women are now warranted.

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