[CANCER RESEARCH (SUPPL.) 42, 3345s–3348s, August 1982]
0008-5472/82/0042-0000$02.00

In Vivo Effects of Δ^1-Testololactone on Peripheral Aromatization[1]

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Abstract

To evaluate the *in vivo* effect of Δ^1-testololactone on peripheral aromatization, studies were performed on seven postmenopausal women with metastatic breast cancer. Analysis of variance indicated that there were significant increases of circulating androstenedione ($p < 0.05$) and estradiol ($p < 0.001$) during administration of different doses of testololactone. Androstenedione levels were increased with all doses of testololactone tested (50, 100, 250, and 500 mg every 6 hr for 14 days each), while estradiol rose with only the 250- and 500-mg dosages. With administration, there was a significant decrease of estrone ($p < 0.001$) with the mean level falling from 26 ± 3 (S.E.) to 11 ± 2 pg/ml. The addition of adrenal suppression (dexamethasone, 1 mg nightly at 11 p.m.) significantly lowered androstenedione ($p < 0.05$) but had no effect on estrone or estradiol levels. Long-term therapy (up to 6 months) with the 250-mg dosage showed continual suppression of estrone with no escape being observed. Studies to determine the reason for the increase of estradiol with testololactone suggested cross-reactivity of the antibody with *in vivo* metabolites of the drug. However, these possible metabolites did not bind to uterine cytosol estrogen receptors. The decrease in estrone with testololactone administration presumably explains its antitumor properties.

In postmenopausal women, the primary source of circulating estrone is the peripheral aromatization of an adrenal androgen, androstenedione (7, 14), while the major source of estradiol is the peripheral conversion of estrone (9). Direct glandular secretion of either estrogen by the adrenal glands or ovaries is minimal (2, 6, 8). Compounds which inhibit peripheral aromatization should lower endogenous estrogen levels and would be of clinical interest in the treatment of conditions such as metastatic breast cancer (11). Δ^1-Testololactone has been shown to inhibit placental aromatization *in vitro* (12, 15, 16) and to lower the peripheral conversion of androstenedione to estrone and to lower estrone but not estradiol levels *in vivo* in postmenopausal women with metastatic breast cancer (3). The present study was undertaken (*a*) to determine the dose-response relationship of testololactone administration, (*b*) to evaluate the dual effects of testololactone administration and adrenal suppression, and (*c*) to examine the long-term action of the drug on endogenous estrogen levels.

Materials and Methods

Seven patients with metastatic carcinoma of the breast agreed to the study protocol and gave written informed consent. All had undergone a surgical or spontaneous menopause. This was supported by the observations that endogenous estradiol levels in all subjects were in the postmenopausal range (<20 pg/ml). All studies were performed in the Surgical Oncology Outpatient Care Facility at University Hospital, University of California at San Diego. Each patient underwent 3 studies. (*a*) The women were given increasing doses of testololactone p.o. The dosages tested were 50, 100, 250, and 500 mg with each dosage being taken every 6 hr for 14 days. (*b*) The subjects were given testololactone (250 mg every 6 hr) and dexamethasone (1 mg each night at 11 p.m.) for 14 days. (*c*) The women were given testololactone (250 mg every 6 hr) for 6 months or until exacerbation of their breast tumor.

Blood samples were drawn before and at the end of each treatment cycle or at the end of each month of long-term therapy. At each sampling period, four 10-ml blood samples were drawn at 15-min intervals beginning at 8 a.m. for measurement of serum androstenedione, estrone, estradiol, and testosterone levels.

Serum androstenedione, testosterone, estrone, and estradiol levels were measured in all samples by radioimmunoassay procedures published previously (1, 4). The tube sensitivities of these assays were 10.4, 2.3, <2, and <2 pg for the respective hormones. To assure that the assays would be able to measure the anticipated low levels of estrogens, 3-ml serum samples were assayed. The mean of the values measured in the 4 samples drawn from a subject on a given day was used as the hormone level in that patient.

The dose-response study was evaluated by analysis of variance. Comparison of the effects of testololactone and testololactone plus dexamethasone was performed by the paired Student's *t* test. Evaluation of the long-term effect of testololactone was also performed by analysis of variance.

Results

Chart 1 shows the mean ± S.E. levels of androstenedione, testosterone, estrone, and estradiol before and during different doses of testololactone. Analysis of variance indicated that there were significant increases of androstenedione ($p < 0.05$) and estradiol ($p < 0.001$) with drug administration. An increase of testosterone was also noted, but this was not statistically significant. For androstenedione, the increase was seen with all dosages, while only the 250- and 500-mg dosages were associated with increases of circulating estradiol. For estrone, there was a significant decrease ($p < 0.001$) with administration with the level falling from 26 ± 3 to 11 ± 2 pg/ml. The decrease was progressive and was maximal with the 250-mg dosage.

Chart 2 compares the base-line levels of androstenedione, testosterone, estrone, and estradiol with the values measured during the administration of 250 mg testololactone every 6 hr with and without dexamethasone. The addition of dexamethasone resulted in a significant decrease ($p < 0.05$) of androstenedione from 566 ± 129 to 380 ± 94 pg/ml. The addition of dexamethasone also lowered the mean testosterone level, but this change was not statistically significant. The addition of dexamethasone had no demonstrable effect on estrone or

[1] Presented at the Conference "Aromatase: New Perspectives for Breast Cancer," December 6 to 9, 1981, Key Biscayne, Fla. This work was supported by USPHS Grant CA23093.

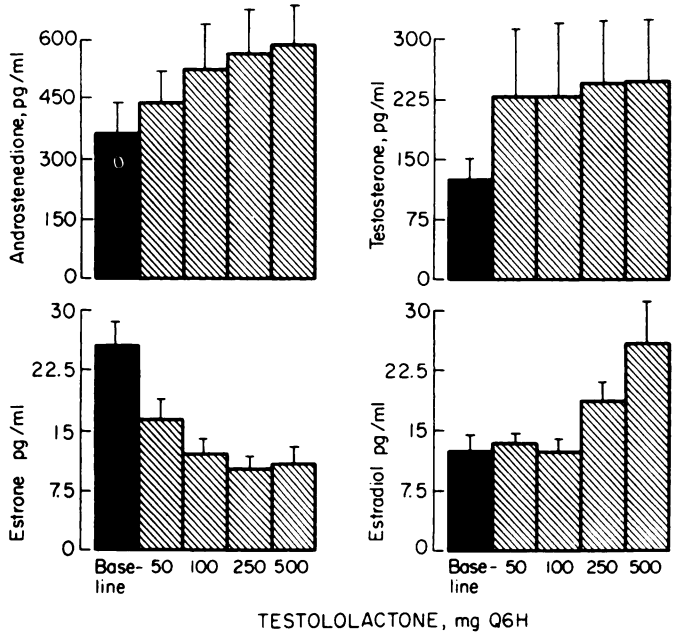

Chart 1. Serum androstenedione, testosterone, estrone, and estradiol levels in 7 postmenopausal women with metastatic breast cancer on different doses of testololactone. *Q6H*, every 6 hr; *bars*, S.E.

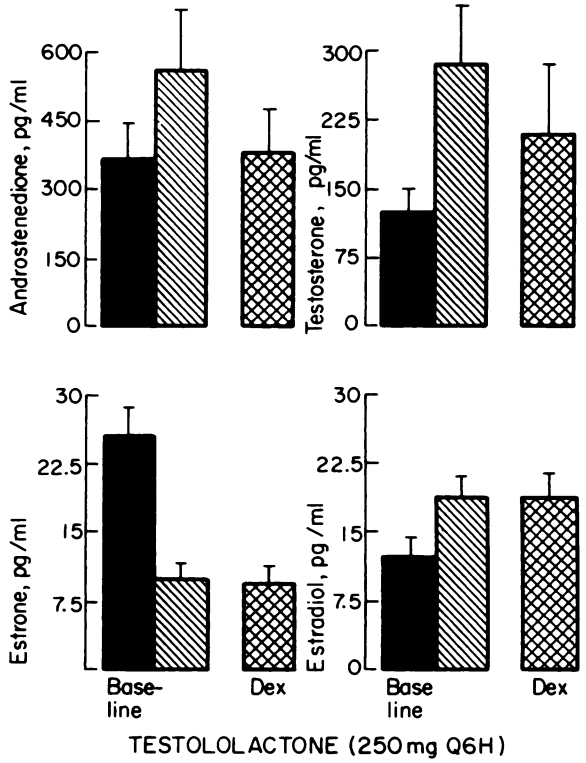

Chart 2. Serum androgens and estrogen before and during testololactone administration with and without dexamethasone (*DEX*). *Q6H*, every 6 hr; *bars*, S.E.

estradiol levels.

Chart 3 compares the mean levels of estrogens before and during long-term therapy (250 mg every 6 hr). Six subjects took the medication for 3 months, 4 subjects took the medication for 3 months, and 3 subjects took the medication for 6 months. In each case, the medication was discontinued be-

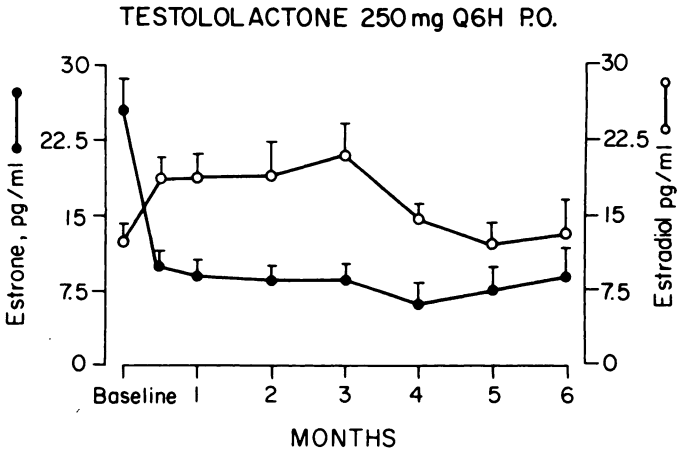

Chart 3. Serum estrone and estradiol levels before and during 6 months of testololactone administration. *Q6H*, every 6 hr; *bars*, S.E.

cause of exacerbation of the disease. After 3 months, the levels of both estrogens were similar to the concentrations observed after 14 days of therapy. The small, apparent decrease of estradiol from 3 to 6 months of therapy was related to the drop out of subjects from the study during those months of treatment.

The question was raised if the apparent rise of serum androstenedione, testosterone, and estradiol with testololactone administration could be the result of cross-reactivity of the drug with the immunoassays used to measure these hormones. To examine this question, 5 samples of a testololactone saline solution (50 μg/ml) were assayed for androstenedione, testosterone and estradiol. The samples were extracted, chromatographed, and assayed in the usual manner. A 0.000002% or less cross-reactivity of the drug was observed for the androstenedione, testosterone, and estradiol assays. Thus, there would have to be between 6000 and 11,000 μg of testololactone per ml in the plasma to account for the apparent elevations of circulating androstenedione, testosterone, and estradiol concentrations observed with the 500-mg dosage. In women, the blood volume is approximately 66 ml/kg. If it is assumed that there is complete absorption of the p.o. administered drug with compartmentalization only in the plasma, then the 500-mg dose would result in approximately a 210-μg/ml plasma concentration in a 60-kg woman with a hematocrit of 40. Thus, it is very doubtful that the minute cross-reactivity of the compound with the androstenedione and testosterone immunoassays could have accounted for the increases of both androgens during drug administration. However, it is possible that cross-reactivity with the estradiol antibody could have accounted for part of the apparent estradiol rise.

To determine if the apparent increase of estradiol could be from cross-reactivity of *in vivo* metabolites of testololactone with the estrogen antibody and if these possible metabolites were biologically active, blood was taken from a subject on the 500-mg dosage. The serum was extracted with ether, and steroids were separated by HPLC.[2] In 0.33-ml fractions of the HPLC eluate, the immunoreactive estrogens were measured using an antibody with a high cross-reactivity to known estrogens found in biological samples. The potential biopotency of circulating estrogens was measured in terms of competition of circulating steroids with tritiated estradiol for binding by rat

[2] The abbreviation used is: HPLC, high-pressure liquid chromatography.

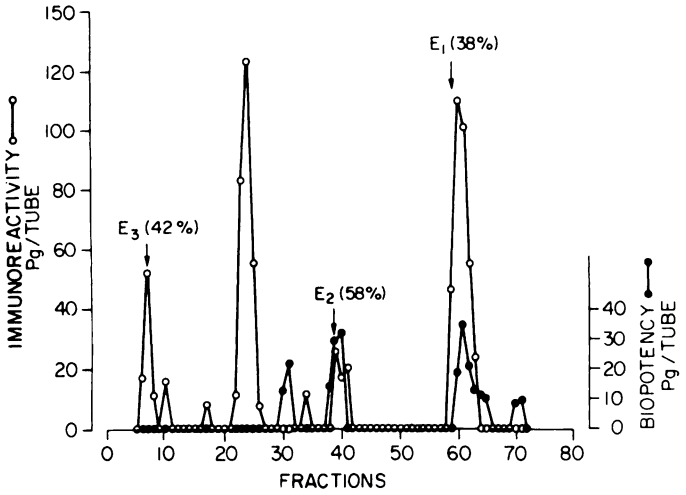

Chart 4. HPLC of serum from a woman on 500 mg testololactone given every 6 hr. Collection of column eluate, 0.3 min/tube; flow rate, 1 ml/min. Measurement of immunoreactive and cytosol receptor binding (biopotency) was performed on all elution fractions. *Arrows*, fractions containing tritiated estrone (E_1), estradiol (E_2), and estriol (E_3) which were added to serum sample prior to extraction and chromatography. Numbers in parentheses, percentage of recovery of major estrogens.

uterine cytosol estrogen receptor. Relative retention times and immunoreactive peak:receptor-binding ratios across a column elution allowed characterization of all estrogenic components. This method has been described in detail previously (5).

Chart 4 shows the amounts of immunoreactive and receptor-reactive estrogens in the HPLC column eluate of the serum from the patient on the 500-mg dosage of testololactone. The elution fractions containing estrone, estradiol, and estriol were located by measurement of tritiated hormonal markers, which had been added to the serum sample before extraction. It is noteworthy that the receptor-reactive peaks were confined mainly to the estrone and estradiol peaks, while several peaks measured by immunoassay, including the largest one were not confined to the estrone, estradiol, or estriol peaks. These extra peaks of immunoreactive estrogen had no receptor binding.

Discussion

These studies showed that testololactone administration is associated with a pronounced decrease of serum estrone levels in postmenopausal women with metastatic breast cancer. This occurred with all doses tested. The lowest dose that resulted in maximal suppression was 250 mg given every 6 hr. Suppression exerted with this dosage lowered the mean estrone level to a value similar to that observed in oophorectomized and adrenalectomized subjects (11). The addition of adrenal suppression (1 mg dexamethasone each night) with testololactone did not result in a greater reduction of estrone levels than did testololactone alone. This dose of dexamethasone was chosen because it suppresses completely the nightly increase of cortisol.[3] Long-term suppression of estrone (up to 6 months) was achieved with testololactone administration, and no escape from suppression was noted.

This fall of estrone levels with testololactone resulted presumably from decreased peripheral conversion of androstenedione to estrone, which has been shown previously by our group (3). This effect of testololactone seems specific since changes in the metabolic clearance rate of androstenedione or the peripheral conversion of androstenedione to testosterone did not occur (3).

Although the above mechanism is the explanation most likely, there are several other possibilities that could account for the decrease in circulating estrone. (*a*) Testololactone could inhibit direct glandular secretion of estrone. This is doubtful since direct secretion of estrone by the adrenal or postmenopausal ovary is very limited (2, 6, 8). (*b*) The drug could reduce the production rate of androstenedione. This is also doubtful but cannot be eliminated because of the potential problems of cross-reactivity of testololactone or one of its metabolites with the androstenedione antibody. Finally, (*c*) the reduction of estrone could occur because of increased clearance rather than decreased production of the estrogen. This is also doubtful but has not been ruled out and is the subject of current research.

The exact mechanism responsible for the inhibition of peripheral aromatization by testololactone has not been established. Siiteri *et al.* (14, 15) and Schwarzel *et al.* (12) have examined the ability of numerous compounds, including testololactone, to inhibit placental aromatization. These compounds appear to inhibit the process by binding to placental microsomal cytochrome P-450, the enzyme system that is essential for placental aromatization of androstenedione. A similar mechanism may be involved with the inhibition of peripheral aromatization.

One of the problems with the present study was the statistically significant increases of androstenedione, and estradiol and the rise of testosterone, which occurred with testololactone administration. Again, there are several potential explanations for these findings. (*a*) Testololactone could reduce the metabolic clearance rate of these 3 steroids. This is unlikely since the metabolic clearance rate of androstenedione is unchanged with administration of the compound (3). (*b*) The production rate of each steroid could be increased either by stimulation of direct glandular secretion or enhancement of peripheral conversion from precursor steroids. Again, this is unlikely but has not been ruled out at the present time. (*c*) There may be cross-reactivity of testololactone or its *in vivo* metabolites with the immunoassays used to measure these 3 steroids. The experiments to determine cross-reactivity of testololactone itself with the steroid immunoassays did not support this possibility, particularly for androstenedione and testosterone. A more probable explanation is that *in vivo* metabolites of testololactone bind to the steroid antibodies giving artificially high readings of circulating androstenedione, testosterone, and estradiol. The presence of several peaks of immunoreactive estrogen, which were not estrone, estradiol, or estriol, in the HPLC column eluate of the serum from a woman on 500 mg testololactone supports this concept. To date, we have studied 11 postmenopausal women who were not taking testololactone, and none had these extra peaks.

Of critical importance to this study is the biological activity of any metabolite of testololactone which cross-reacts with the estradiol immunoassay. Although direct evidence is lacking, there are indirect data which suggest that the possible metabolites have limited biological activity. Comparison of the ratios of immunoreactor:radioreceptor binding of the HPLC eluate

[3] H. L. Judd, unpublished data.

revealed that none of the extra immunoreactive peaks (including the largest one) had receptor binding. This suggests that these peaks had limited if any estrogenic activity.

In summary, data are presented that indicate testololactone administration is associated with a marked decrease of circulating estrone. This fall may explain the antitumor properties of this compound (13). Rises of androstenedione, testosterone, and estradiol were observed with the administration of the compound and were presumed to represent cross-reactivity of testololactone or its *in vivo* metabolites, with the immunoassays used to measure these hormones. For estradiol, the presumed metabolites appeared to have immunological activity but limited if any biological action.

Acknowledgments

We wish to thank L. Obnial and M. A. Lu for their expert technical assistance and M. Greenstein for her secretarial expertise.

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