Antitumor Effect of Two Oral Steroids, Mepitiostane and Fluoxymesterone, on a Pregnancy-dependent Mouse Mammary Tumor (TPDMT-4)\textsuperscript{1}

Akio Matsuzawa and Tadashi Yamamoto

Laboratory Animal Research Center [A. M.] and Department of Oncology [T. Y.], Institute of Medical Science, University of Tokyo, P. O. Takanawa, Tokyo 108, Japan

SUMMARY

Pregnancy-dependent TPDMT-4 mammary tumors, characterized by requiring estrogen, progesterone, and pituitary hormones for growth, grew continuously in female DDD mice carrying pituitary isografts. The experimental model was used to investigate the antitumor effects of two p.o. steroids, mepitiostane and fluoxymesterone. When tumors implanted with pituitary isografts into the fat-pad reached palpable size, animals received 6 doses/week of 0.1, 0.3, 1.0, and 3.0 mg of either steroid intragastrically. Mepitiostane significantly suppressed tumor growth with regression in 25 and 29% of animals at 1.0 and 3.0 mg, respectively, but had no inhibitory effects at other doses. Fluoxymesterone retarded tumor growth during the first week of treatment at 3.0 mg but finally had no inhibitory effects at any doses. Under similar conditions ovariectomy caused tumor regression immediately, and epitostanol, the parent steroid of mepitiostane, significantly suppressed tumor growth when given in 3 injections/week of 0.5 mg s.c. Tumors had papillary structures and almost lacked secretory activity. A comparison of these findings to those obtained with earlier generations suggests that TPDMT-4 tumors became less sensitive to the antitumor effect of epitostanol and were able to grow at lower hormone levels with succeeding generations. Morphologically, progression to more cancer and less secretory activity was noticed.

INTRODUCTION

A very stable, transplantable pregnancy-dependent mammary tumor line designated TPDMT-4 was first successfully isolated in DDD mice, a strain of European origin (8). TPDMT-4 tumors are characterized by growth during pregnancy, rapid regression after parturition in breeders, and virtually no growth in virgins. These mammary tumors are very similar to the normal mammary gland in their response to a variety of hormones. Estrogen and progesterone secreted from the ovary (9) and pituitary hormones including prolactin (11) are essential for TPDMT-4 tumor growth. The tumors have both cytoplasmic and nuclear estrogen receptors. TPDMT-4 tumors grew without regression in syngeneic female mice carrying ectopic pituitary isografts. Pituitary glands released from hypothalamic control secreted primarily prolactin, with both luteotropic and mammotrophic effects (17). Continuous tumor growth also occurred in virgins given implants of a pellet containing 17β-estradiol and progesterone (9). These experimental systems have provided useful models for studies on endocrinotherapy of hormone-dependent mammary tumors. In hosts with ectopic pituitary isografts, the tumors ceased to grow and began to regress immediately after treatment with epitostanol [2α,3α-epitho-5α-androstan-17β-ol (10275-S)] (10). The steroid also suppressed mammary gland development in the hosts. The steroid has been used with some efficacy for treatment of advanced breast cancer (3). A derivative of the steroid that exerts an antiestrogenic effect after p.o. administration was synthesized (5); the derivative is mepitiostane [2α,3α-epitho-5α-androstan-17β-yl 1-methoxy-cyclopentyl ether (10364-S)]. Mepitiostane has been tried with some efficacy in advanced human breast carcinomas (6). Fluoxymesterone or 9α-fluoro-11β, 17β-dihydroxyandrost-4-en-3-one is another p.o. steroid that has been extensively used for treating human breast carcinomas (4, 7, 20). Both mepitiostane and fluoxymesterone antagonized the uterine weight gain and vaginal cornification produced by exogenous estrogen in immature or ovariectomized mice, but only mepitiostane inhibited ductal growth of the mouse mammary gland and growth of hormone-dependent rat mammary tumors (15).

This study was conducted to investigate the antitumor effects of mepitiostane and fluoxymesterone on TPDMT-4 mammary tumors in pituitary isograft-bearing hosts. Treatments with epitostanol and ovariectomy were included to examine possible changes in TPDMT-4 tumors in response to hormones during the course of serial transplantations.

MATERIALS AND METHODS

Animals. DDD mice, bred and maintained in the mouse colony in the Laboratory Animal Research Center, were used in all experiments. Female mice, 6 to 7 weeks old, served as recipients. Pituitary isografts were obtained from syngeneic male and female mice 2 to 4 months old. The origin and some characteristics of the strain have been described in detail elsewhere (12). DDD mice originated in Europe, carry a virulent strain of murine mammary tumor virus (MTV-P), and are characterized by rare and late development of mammary tumors. Under the present exper-
Tumors. TPDMT-4 is a transplantable pregnancy-dependent mammary tumor line established from a spontaneous mammary tumor of a DDD mouse. The response to hormones and other properties of this particular tumor line have been described in detail (8, 9, 11). A tumor at transplant generation 23 or 24 was obtained from a female, close to termination of her second pregnancy, and was cut into fragments measuring approximately 2 x 2 x 2 mm. A tumor fragment and 3 pituitary isografts were implanted into the right inguinal fat-pad of each recipient. These animals were inspected, and the 2 diameters, bisecting palpable tumors at a right angle to each other, were determined twice weekly. The arithmetic mean of the 2 diameters was designated as the tumor diameter. Growth curves were obtained by charting the mean diameters versus time for each group.

Treatments. In Experiment 1 the antitumor effect of mepitiostane and fluoxymesterone was examined at daily doses of 0.1, 0.3, and 1.0 mg. The experiment was carried out at transplant generation 23. When tumor sizes had reached 4 x 4 mm to 11 x 11 mm 27 to 30 days after implantation, the animals were randomly divided into 7 groups of 14 animals each; no significant difference in tumor diameters, pretreatment periods (intervals between tumor implantation and the start of treatments), and latent periods (intervals between tumor implantation and the appearance of palpable tumors) was noticeable among them. Each test dose of steroids was dissolved or suspended in 0.1 ml sesame oil and was p.o. administered with a stomach tube 6 times weekly over a treatment period of 36 days, starting on the day of animal grouping. Control animals received i.g. 2 doses of sesame oil alone.

Experiment 2 was carried out at transplant generation 24 to examine antitumor effect of both steroids at a higher daily dose of 3.0 mg. Treatment with 1.0 mg of either steroid was included for reference because the growth of tumors varied to some degree with the tumors that served as sources of tumor grafts (10). When tumor sizes reached 3 x 3 mm to 12 x 12 mm after 27 to 32 days, animals were randomly divided into 5 groups of 15 animals each as described in Experiment 1. Treatments were then performed for 35 days in the same manner and at the same frequency as in Experiment 1.

In Experiment 3 the antitumor effect of epitiostanol and ovarioectomy on TPDMT-4 tumors was investigated at transplant generation 24. When tumor sizes reached 3 x 3 mm to 6 x 6 mm after 25 days, animals were randomly divided into 5 groups of 7 animals each; no significant difference in tumor diameters and latent periods was noted. A group of animals was ovarioctomized immediately. Another group was treated with epitiostanol during a 41-day period commencing on the day of animal grouping. Epitiostanol (0.5 mg) was suspended in 0.1 ml of an aqueous solution containing 0.9% (w/v) NaCl solution, 0.4% (v/v) polysorbate 80, 0.5% (w/v) carboxymethylcellulose, and 0.9% (v/v) benzyl alcohol and was injected s.c. into the neck region of the back 3 times weekly. Control and ovarioctomized animals received s.c. injections of the vehicle alone at the same frequency during the same period. In all experiments the first treatment day was designated as Day 0. At the termination of treatments, all animals were killed to obtain the weights of the body, ovaries, uterus, adrenal glands, and thymus.

Morphology. In Experiments 1 and 2 the tumors and ovaries were fixed in 10% formalin, processed routinely, and stained with hematoxylin and eosin for histological study. The whole skins from all control and experimental animals were fixed in 10% formalin, and whole mounts of the third mammary glands were prepared as described elsewhere (13, 14). The extent of lobuloalveolar development was classified into 5 grades numbering 0 to 4, according to the criteria described in "Results."

Statistics. The significance of difference in tumor diameter and tumor, body, and organ weights between each treated group and the corresponding control was examined by Student's t test. The differences were evaluated as significant at \( p < 0.05 \).

RESULTS

Experiment 1. Antitumor Effect of Mepitiostane and Fluoxymesterone at Small Doses

Each group included 14 animals with palpable tumors at the commencement of treatment. However, 1 animal in each group was discarded due to unsuccessful intubation in all groups except for those treated with 0.1 and 0.3 mg fluoxymesterone in which groups all animals were successfully treated throughout the experimental period. As indicated in Table 1, mepitiostane at doses of 0.1 and 0.3 mg and fluoxymesterone at all doses had no influence on tumor weights and diameters. Although it is not illustrated in the charts, the growth curve of the tumor followed virtually the same course in these groups as in the control. Mepitiostane significantly suppressed tumor growth as reflected by weight and diameter at a dose of 1.0 mg (Table 1). Tumor regression occurred in 2 animals of this group (Chart 3). The degree of tumor growth retardation resulting from this dose was similar to that obtained at the same dose in Experiment 2 and is presented in Chart 1.

Experiment 2. Antitumor Effect of Mepitiostane and Fluoxymesterone at Larger Doses

Each group included 15 animals with palpable tumors at the start of treatment. However, 1 animal in each group was discarded due to failure in intubation during the treatment period in all groups except in the control group and the group treated with 1.0 mg mepitiostane. At both doses mepitiostane gave rise to the same significant suppression of tumor growth (Table 1; Chart 1). Tumor regression occurred in 5 and 4 animals receiving 1.0 and 3.0 mg mepitiostane, respectively (Chart 3). It also occurred in 1 animal of the group treated with 1.0 mg fluoxymesterone. However, there was no significant difference in mean tumor...
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**Experiment 3. Antitumor Effect of Epitiostanol and Ovariotomy**

Experiment 3 was conducted to investigate the possible variation in response of the TPDMT-4 tumor to both treatments during serial transplantsations. At the eighth transplantation generation the tumors ceased to grow and began to regress immediately after ovariotomy or after the start of epitiostanol treatment at daily doses higher than 0.1 mg (10). In the present observation carried out at the 24th generation, ovariotomy also gave rise to immediate tumor regression in all animals (Chart 2). In contrast, 3 injections/week of 0.5 mg epitiostanol s.c. did not cause tumor regression in any animals, although it suppressed tumor growth to a significant degree (Table 1; Chart 2). These facts show that the tumor had become less sensitive to the
diameter and weight between this group and the control as evidenced in Experiment 1 (Table 1; Chart 1). At a higher dose, fluoxymesterone inhibited tumor growth especially during the first week of treatment, although in the end the inhibitory effect did not reach a significant level.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean tumor diameter (mm)</th>
<th>Mean tumor weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (7)</td>
<td>27.7 ± 0.7</td>
<td>33.1 ± 1.2</td>
</tr>
<tr>
<td>0.5 mg Ep. (7)</td>
<td>26.9 ± 0.3</td>
<td>35.3 ± 1.2</td>
</tr>
<tr>
<td>Mean = S.E.</td>
<td>Number = S.E.</td>
<td>Significant difference (p &lt; 0.05) compared with the control group.</td>
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**Chart 1. Effect of mepitiostane and fluoxymesterone on growth of TPDMT-4 mammary tumors (Experiment 2).** O, control; D, 0.1 mg mepitiostane; ●, 1.0 mg mepitiostane; ▲, 3.0 mg mepitiostane; △, 3.0 mg fluoxymesterone; bar, S.E.

**Chart 2. Effect of epitiostanol and ovariotomy on growth of TPDMT-4 mammary tumors (Experiment 3).** O, control; □, 0.5 mg epitiostanol 3 times weekly; △, ovariotomized; bar, S.E.
antitumor effect of epitiostanol between transplant generations 8 and 24.

Effect of Mepitiostane and Fluoxymesterone on Body and Organ Weights, Mammary Gland Development, and Ovary and Tumor Morphology

Body and Organ Weights (Table 1). Body weight was significantly increased in groups given 1.0 and 3.0 mg mepitiostane and 3.0 mg fluoxymesterone. Uterine weight gain was significant in the groups treated with 0.3 to 3.0 mg mepitiostane and 3.0 mg fluoxymesterone, although the reason for this is unclear. Both steroids gave rise to a significant decrease in ovarian weight at all of the doses tested. The decrease was especially remarkable in the groups receiving 1.0 and 3.0 mg mepitiostane, in which significantly suppressed tumor growth and conspicuous degeneration of luteal elements of the ovary were observed. Although significant weight loss of the thymus occurred in all treated groups, this phenomenon was far more striking in the mepitiostane group. The particular action spectrum of mepitiostane on these parameters is very similar to that observed in its parent steroid, epitiostanol (10).

Mammary Gland Development. After the observation of all whole-mount preparations, the following criteria were established to classify the extent of lobuloalveolar development into 5 classes: 0, the presence of end buds and the absence of alveoli; 1, the presence of a few alveoli and few lobules (Fig. 1); 2, the presence of many alveoli and a few small lobules (Fig. 2); 3, the presence of a considerable number of small and developed lobules (Fig. 3); 4, the presence of highly developed lobules or complete lobuloalveolar development (Fig. 4). The gland of Grade 4 is significantly increased in groups given 1.0 and 3.0 mg mepitiostane, and 3.0 mg fluoxymesterone, although in about 40% of animals treated with 3.0 mg fluoxymesterone. Significantly, lower tumor weight was recorded only in the first 2 groups (Table 1). Tumor regression was mostly observed in animals with virgin mammary glands (Figs. 1 and 2), although it noticeably occurred in a few animals with mid- to late-pregnant glands. In general the extent of mammary gland development was positively correlated with tumor weight as previously reported (9, 10).

Ovary Morphology. In the pituitary isograft-bearing controls, the ovaries had many apparently active corpora lutea and follicles of various developmental stages (Fig. 6). The ovaries, although significantly lower in weight, showed similar morphology in the groups treated with 0.1 mg mepitiostane and 0.1 or 0.3 mg fluoxymesterone. Stronger degeneration of luteal components and more decrease in the number of solid, active corpora lutea occurred at higher doses in both steroids (Fig. 7). In particular no apparently active corpora lutea were found in 3 animals receiving 3.0 mg fluoxymesterone and in 19 and 12 animals administered 1.0 and 3.0 mg mepitiostane, respectively (Fig. 8). Folliculogenesis was not markedly disturbed in any of these animals except for 1 receiving 3.0 mg mepitiostane. The significant decrease in ovary weight observed at all test doses of both antiestrogens (Table 1) is attributable to their selective effect on luteal components. Epitiostanol, the parent compound of mepitiostane, also had a primary effect on these components of the ovary (10).

Tumor Morphology. Tumors from the pituitary isograft-bearing controls were composed of small cuboidal epithelial cells. The cells were arranged in 2 or more layers and lined angular spaces or clefts in most areas (Fig. 9). These epithelial strands were separated by a small amount of connective tissue and occasionally had papillary projections into the spaces (Fig. 10). In minor areas tubular structures were formed by cells of the same type arranged in single rows (Fig. 11). However, tumors had no obvious glandular and acinar structures, and most lumina and spaces formed were devoid of secretion or fluid (Figs. 9 to 11). In this respect they presented a sharp contrast to the tumors at earlier generations, which grew under the same hormonal condition and were characterized by predominant glandular and acinar structures with the presence of secreted material in lumina (9, 10). No especially noticeable morphological changes occurred in any of the treated animals. Thus, the peculiar cystadenomatous structure with much secreted material induced by either epitiostanol or testosterone at earlier generations (10) was not observed after treatment with epitiostanol, mepitiostane, or fluoxymesterone at generations 23 and 24 in the present study. These findings suggest that the tumor cells may have altered their response to hormones and secretory activity.
This study has focused on antitumor effect of both p.o. efficacy for treatment of advanced breast cancer (4, 7, 20). The new synthesized to obtain the antitumor effect p.o. (5). The new cancers (3). Mepitiostane, a derivative of epitiostanol, was tumor activity on hormone-dependent rat and mouse mammary tumors. Pituitary grafts free from hypothalamic control gave rise to clinical remission in about one-fourth of the advanced breast carcinomas (6). Fluoxymesterone is an p.o. effective, highly potential androgen and has been used with some efficacy for treatment of advanced breast cancer (4, 7, 20). This study has focused on antitumor effect of both p.o. steroids on the TPDMT-4 tumor. Emphasis also was placed on progression of the tumor. TPDMT-4 is a transplantable pregnancy-dependent mammary tumor line characterized by its requirement for estrogen, progesterone, and pituitary hormones for growth and by growth during pregnancy and rapid regression after parturition (8, 9, 11). It can grow without regression in pseudopregnant mice bearing pituitary isografts. Pituitary grafts free from hypothalamic control gave rise to tumor growth and lobuloalveolar development of host mammary glands by producing primarily prolactin, with a luteotrophic effect on the ovary and a mammatropic effect on the mammary gland (10, 17). In this experimental model, mepitiostane suppressed the tumor growth to a significant degree at daily doses of 1.0 and 3.0 mg (Table 1; Chart 1), with tumor regression in some animals (Chart 3). In contrast, fluoxymesterone caused moderate but insignificant retardation of tumor growth at a daily dose of 3.0 mg (Table 1; Chart 1). As in the previous observations (9, 10), there was positive parallelism between tumor growth and mammary gland development; tumor weight tended to be larger in animals with more highly developed mammary glands (Chart 3). At a dose of 1.0 mg, mepitiostane inhibited both tumor growth and mammary gland development, but fluoxymesterone exhibited no influence on either of them. In addition, the former suppressed estrogen-stimulated mammary duct growth, whereas the latter did not exhibit this effect (15). These findings are very interesting because this particular tumor line was thought to be closely related with ductal cells in its origin (11). The action spectrum of mepitiostane on body and organ weights (Table 1) and on ovary morphology (Figs. 7 and 8) is very similar to that of epitiostanol (10). Especially marked atrophy of the thymus and degeneration of luteal elements of the ovary are specific to both steroids, supporting the hypothesis that mepitiostane may manifest its activity after it has reverted to epitiostanol in the body. Recently, this reaction has been proven to occur in the body after p.o. administration (H. Yoshida, unpublished data).

Mepitiostane has been demonstrated to have androgenic, antiestrogenic, anabolic, and myotrophic activities (15, 16). These activities were reflected in body weight gain, thymic atrophy, uterine weight gain, adrenal involution, and suppressed mammary tumor development (Table 1; Chart 3). It is difficult to determine which of the activities was most closely related to the antitumor effect in the experimental model. However, as evidenced in epitiostanol (10), mepitiostane might inhibit tumor growth through partial suppression of ovary hormone production and direct action on tumor cells, phenomena that are ascribable to its antiestrogenic and androgenic activities. With regard to its direct action, epitiostanol, believed to be an active form of mepitiostane in vivo, competitively inhibited the binding of 17β-estradiol to cytoplasmic estrogen receptors from the tumors, but fluoxymesterone did not (T. Hori and A. Matsuzawa, unpublished data).

Ovariectomy gave rise to immediate regression of TPDMT-4 tumors in the present experiment (Chart 2) as in the previous one (10). As for epitiostanol, however, the result was inconsistent in both experiments. The steroid caused tumor regression at the 7th and 8th transplant generation (10), but it only suppressed tumor growth at transplant generation 24 (Chart 2). This discrepancy can be explained by progression of the tumor cells toward decreased sensitivity to the antitumor effect in the course of successive transplants. The morphological evidence also supports this explanation. Tumor morphology varied with generations, although individual cells have remained of the same cuboidal epithelial type. Tumors were primarily composed of glandular and acinar structures with strong secretory activity at earlier generations (9, 10). In contrast they consisted mainly of papillary structures with little secreted material and cells arranged in multiple layers resulting in lack of glandular and acinar formation at later generations (Figs. 9 to 12) and appeared morphologically more malignant. However, the content of cytoplasmic estrogen receptors determined by the dextran-coated charcoal assay has not changed during this period of time (A. Matsuzawa, T. Hori, and T. Suzuki, unpublished data).

The TPDMT-4 tumor line since its isolation has been maintained on the criterion of pregnancy-dependent growth in breeders and no appreciable growth in virgins. This condition had been met at least up to generation 23 (11) at which these experiments were carried out. At generation 27 tumor cells remained dormant and did not produce significant outgrowths during a period of 3 months in the 6 virgins observed. However, tumors grew sporadically in some virgins at the transplant generation 28 and thereafter. In fact, significant tumor growth occurred in 3 of 7 virgins at generation 29; their sizes reached 4 x 7 mm, 4 x 7 mm, and 7 x 8 mm, respectively, 2 months after implantation. More significantly, these tumors grew irregularly although not linearly and regressed occasionally. In breeders, tumors even have grown during pregnancy and regressed after parturition. Even at earlier generations the tumor was able to grow to a small although appreciable size in DDD x C3H virgins (A. Matsuzawa, unpublished data), which are probably different from DDD virgins in the levels of hormones that regulate tumor growth. These observations suggest that the TPDMT-4 tumor has gradually progressed so that it is able to grow at lower hormone levels and that it will be autonomous finally. Response of tumors grown in virgins to ovariectomy and hormones is under investigation.

Many investigators have reached the consensus that hormone-dependent tumors show progression to autonomy (2, 18, 19, 21). Generally, the progression occurs rapidly in serial transplantations. Tumors were classified as type B according to criteria of Dunn (1) in all animals.
murine tumor virus-induced mouse mammary tumors. Many hormone-dependent mouse mammary tumors have become autonomous in situ or during several successive transplants (2, 21). In this respect TPDMT-4 is a very exceptional mouse mammary tumor. Such a slow progression of hormone-dependent tumors to autonomy was reported in rats (18, 19). A series of studies on TPDMT-4 tumors (9-11) including this one indicate the difficulty of predicting what type of endocrine therapy will be most effective against a hormone-dependent breast cancer.

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REFERENCES

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