The Selective Estrogen Receptor Modulator Trioxifene (LY133314) Inhibits Metastasis and Extends Survival in the PAIII Rat Prostatic Carcinoma Model


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ABSTRACT

Trioxifene (LY133314) is a selective estrogen receptor modulator (SERM) with competitive binding activity against estradiol for estrogen receptor α (ERα) and antagonistic activity against ERα-mediated gene expression. The PAIII rat prostatic adenocarcinoma (PCa) is an androgen receptor-negative, ERα- and ERβ-positive, spontaneously metastatic rodent tumor cell line. After s.c. implantation of 10^6 PAIII cells in the tail, s.c. administration of trioxifene (2.0, 4.0, 20.0, or 40.0 mg/kg-day) for 30 days produced significant (P < 0.05) inhibition of PAIII metastasis from the primary tumor in the tail to the gluteal and iliac lymph nodes (maximum nodal weight decreases, 86% and 88% from control values, respectively). PAIII metastasis to the lungs was significantly inhibited by trioxifene treatment. Numbers of pulmonary foci in PAIII-bearing rats were significantly (P < 0.05) reduced by trioxifene administration in a dose-related manner (maximal reduction, 98% from control values). Continual administration of the compound significantly (P < 0.05) extended survival of PAIII-bearing rats. Trioxifene inhibited the proliferation of PAIII cells at micromolar levels in vitro but did not slow growth of the primary tumor growth in the tail. Trioxifene administration also produced regression of male accessory sex organs. In PAIII-tumor-bearing animals, trioxifene administration produced a maximal regression of 76% for ventral prostate and 64% for seminal vesicle (P < 0.05 for both). SERMs may be preferable to estrogens given their efficacy in experimental PCa models and relative lack of side effects observed in clinical trials. Our data support the contention that trioxifene represents a SERM with potential antitumor efficacy for the treatment of androgen-independent, metastatic PCa.

INTRODUCTION

Estrogen administration has long been recognized as an effective hormonal ablative therapy to treat disseminated PCa (1). However, these antitumor effects may result from a centrally mediated decrease in testicular androgen levels. Clinical responses to estrogens such as diethylstilbestrol (Fig. 1a) in human prostatic malignancies may not be entirely attributable to decreased circulating androgens. Doses of synthetic estrogens that are efficacious in treating human PCa do not consistently reduce circulating testosterone to castrate levels (2-4). Indeed, estrogens may exert direct cytoreductive effects through ER (5) in PCa cells that contribute to the observed clinical antitumor activity (6). Although estrogens such as diethylstilbestrol are effective in treating progressive, metastatic PCa, this utility is complicated by the risk of cardiovascular side effects. Consequently, the use of estrogens in treating advanced PCa has been superseded by luteinizing hormone-releasing hormone agonist analogues that produce androgen ablation but do not induce hypercoagulation or increase the incidence of cerebrovascular accidents.

Two scientific developments have raised the possibility of revisiting the use of ER mechanistic-based therapies for treating PCa. First, SERMs possess agonist and antagonist activities that are dependent on the specific tissue type and interactions of a specific agent with ER subtypes (7). Within this pharmacological class, individual SERMs have differing antitumor activities in treating female malignancies. The first-generation SERM, tamoxifen (Fig. 1b) has been widely used to treat and prevent breast cancer and osteoporosis, but clinical experience in treating male disorders is limited. Second, a novel ER gene product, ERβ (also known as ERβ1), was cloned from a rat prostatic cDNA library and is expressed in murine and human prostate (8–10). ERβ has been proposed to act as a ligand-activated tumor suppressor (11). The more recently characterized ER has been designated ERβ, whereas the classical ER characterized in female accessory sex organs is designated ERα.

Trioxifene (LY133314; [3,4-dihydro-2-(4-methoxyphenyl)-1-naphthalenyl][4-[2-(L-pyrrolidinyl)]ethoxyphenylmethanone, methansulfonic acid salt; Fig. 1c) is a SERM with activity in rat uterine bioassays (12). In these estrogen-supplemented female rats, the antagonist activity of trioxifene was comparable to that of tamoxifen. The inherent estrogenicity of trioxifene in immature female rats was approximately one-third less than that of tamoxifen (12). Trioxifene has demonstrated activity in dimethylbenzanthracene-induced mammary carcinoma in rats (13) and human breast cancer patients (14). Our laboratory first demonstrated that SERMs such as LY117018 (Fig. 1d; Ref. 15) and raloxifene (Fig. 1e) produce significant antiprostastic (16) and antitumor responses in male LW rats that had received s.c. injections of PAIII cells in the tail (17). The antitumor activity of SERMs in rat models have recently been extended to cells and tumors of murine (18) and human origin (19). Given the ability to surgically reduce primary tumor bulk and the lack of curative chemotherapy, antitumor modules may be useful to treat disseminated, androgen-independent human PCa after hormonal ablation (20). The PAIII adenocarcinoma in LW rats is a spontaneously metastatic tumor that is useful to evaluate agents to treat disseminated PCa. When PAIII cells are injected s.c. into the tails of male LW rats, a reproducible, time-dependent, sequential spread of the tumor through the gluteal and iliac lymph nodes to the lungs is observed (21). The morphology of the PAIII tumor resembles anaplastic lesions in humans, supporting its utility in evaluating cytotoxic and antitumor agents in advanced disease (22–24). This report characterizes the expression of ERα and ERβ protein in PAIII rat PCa cells as well as the ERα/ERβ selectivity of binding and agonist activity of trioxifene in vitro. Our data show that trioxifene administration dramatically suppresses PAIII metastasis to the lymph nodes and lungs, extends survival, produces involution of male accessory sex organs, and provides additional rationale for the use of SERMs to treat human PCa.

MATERIALS AND METHODS

ERα and ERβ Binding Assay. The ER competition binding assays with purified ERα and ERβ followed modification of previously published proce-
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Fig. 1. Chemical structures of diethylstilbestrol (a), tamoxifen citrate (b), troxifene (LY133314, c), LY171018 (d), raloxifene (LY156758, e), and toremifene (f).

Western Blot Assays. Protein extracts were taken from PAIII cells and analyzed by previously described Western blotting techniques (26). Protein lysates from PAIII, LNCaP, and LNCaP-derived LNAI T1.16 cells (27) were previously prepared with use of radioimmunoprecipitation assay lysis buffer (New England Biolabs, Cambridge, MA). For Western blots, 30–40 μg of protein extract/lane was electrophoresed, transferred to polyvinylidene membranes (Hybond-P; Amersham Pharmacia Biotech) using the XCell II Mini-Cell apparatus (Novex, San Diego, CA), and immunoblotted. The antibodies used in these studies were as follows: ERα (1:250 dilution; Chemicon International, Inc., Temecula, CA), ERβ (1:200 dilution; Upstate Biotechnology, Inc., Lake Placid, NY), AR (1:500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, Biochemistry, CA), and β-actin (1:2000; Sigma). Anti-mouse IgG, anti-rabbit IgG (Santa Cruz Biotechnology), and anti-sheep IgG (Upstate Biotechnology) secondary antibodies were used at a 1:2000 dilution. All Western blots were detected by chemiluminescence (Pierce, Rockford, IL) captured with the Lumi-imager and quantitated using the Lumi-Analyser software (Roche Molecular Biochemicals, Indianapolis, IN).

PI Cell Proliferation Assay. PI (Sigma) for the proliferation assays was stored at a stock concentration of 1.0 mg/ml of H2O (−4°C). Five hundred PAIII cells per well (passage 113–115) in 100 μl of growth medium (DMEM with 10% fetal bovine serum) were plated in each well of a 96-well plate. An additional 100 μl of growth medium containing either troxifene or LY117018 was added to each well at 24 h after plating to give a final incubation volume of 200 μl. Fresh medium containing drug or DMSO control was added every 2–3 days for a total of 7 days. Replacement of the growth medium with 200 μl of PBS and 10-min centrifugation of the plates (2000 rpm) completed the preparation. A working solution of PI was prepared at a final concentration of 50 μg/ml in distilled H2O. Twenty-five μl of working solution was added to each well. Cells were lysed by placing the plates in a −80°C freezer overnight and thawing to room temperature in a 37°C incubator. Foil-covered 96-well plates were placed on a rotator for 30 min and analyzed using the Victor® II fluorescent plate reader (Wallac). The plates were read from the bottom with excitation at 500 nm and emission at 615 nm for 1 s. Raw data were obtained as light units and reported as percentage of control (JMP; SAS Institute, Cary, NC).

PAIII Cell Culture and LW Rats. Dr. Morris Pollard (University of Notre Dame, South Bend, IN) supplied a stock culture of PAIII rat PCa cells at passage 107. This original stock culture was expanded through two passages and stored in liquid nitrogen with 10% DMSO as a cryoprotectant. The cells were kept in liquid nitrogen as 1.0-ml aliquots at a minimum of 1.0 × 106 cells/ml. PAIII cells were grown in antibiotic-free MEM with Earle’s salts (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Hyclone Laboratories, Logan, UT). The cells were grown to confluence and harvested with use of 0.06 units/cm2 trypsin (2× recrystallized grade; Worthington Biochemical Corp., Freehold, NJ).

The breeding stock of LW rats was a gift from Dr. Morris Pollard. LW rats were maintained as a closed colony at Harlan Industries (Cumberland, IN). Male rats weighing 110–125 g were used. Two or three rats were housed in screen-bottomed cages in a light-controlled environment (lights on: 6:00 a.m., lights off: 8:00 p.m.). Water and powdered Rodent Laboratory Chow 5001 (Ralston-Purina, St. Louis, MO) were supplied ad libitum. These investigations were conducted under practices outlined for the care and use of laboratory animals set forth by the NIH and the American Association for Laboratory Animal Care.

On day 0, animals were randomized into five or six groups (10 rats/group). All except one of these groups received injections of PAIII cells. Injections of tumor cells and surgical procedures were done under light Metofane (Pittman-Moore, Washington Crossing, NJ) anesthesia. For each injection, 105 PAIII cells in a volume of 50 μl were injected s.c. into the dorsal surface halfway between the base and tip of the tail with a 25-gauge needle. Control animals received saline injections. Rats not receiving injections of PAIII cells were designated the non-PAIII-bearing (NO PAIII) control group. These studies were conducted for 28–29 days. In the dose–response studies, PAIII-bearing experimental groups were administered troxifene (2.0, 4.0, 20.0, and 40.0 mg/kg) as 0.2-ml single daily s.c. injections. Daily injections were given in the morning (8:00 to 9:00 a.m.). Control groups received equivolumetric vehicle injections.

Troxifene administered to PAIII-bearing rats was dissolved in ethanol and diluted to a final ethanol:peanut oil ratio of 1:10. The compound was stored in lightproof containers at −20°C before formulation and in lightproof bottles at room temperature during compound administration. Troxifene for injection was formulated for 2-week treatment intervals on a body-weight basis. Initial dose formulations were calculated based on previously observed 2-week body weight gains in untreated rats. After 2 weeks of troxifene treatment, doses in each group were adjusted relative to changes in group body weight mean values.

One-half of the rats in each treatment group were sacrificed on day 28. The remaining animals were sacrificed the following day. Rats were sacrificed by
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RESULTS

Trioxifene Binds to rhERα and rhERβ Protein and Inhibits ERα-mediated Luciferase Activity. The competitive activity of trioxifene for [3H]-E2 binding to rhERα and rhERβ binding are depicted in panels a and b of Fig. 2, respectively. Trioxifene competed with [3H]-E2 binding to rhERα with an IC50 of 203.49 nM and a Kd of 20.84 nM. The compound was less potent at displacing [3H]-E2 binding to rhERβ, with an IC50 of 1506.04 nM and a Kd of 144.85 nM.

An ERα- and ERβ-ERE-driven luciferase reporter assay was used to assess the estrogenic activity of trioxifene in PAIII cells. E2 administration induced ERα- and ERβ-ERE-mediated luciferase transcription in cultured PAIII cells at concentrations >10 nm and >100 nm, respectively. Trioxifene was a weak antagonist for ERα-mediated luciferase transcription in PAIII cells, inhibiting reporter activity at concentrations >100 nm (Fig. 3). Trioxifene had no activity against ERβ-ERE-mediated luciferase transcription in PAIII cells at any of the concentrations studied.

PAIII Cells Express ERα and ERβ but Not AR. Cultured PAIII and LNCaP-derived LNAI T1.16 PCa cells expressed ERα (Fig. 4). PAIII cells expressed lower levels of ERα than their human PCa cell line comparators. ERβ expression was detected in PAIII cells, albeit at lower levels than in the parental LNCaP cells. AR protein immunoreactivity was observed in LNCaP but not detected in PAIII rat PCa cells.

Trioxifene Inhibits In Vitro Proliferation of PAIII Cells. PAIII cells grown in culture were exposed to trioxifene at concentrations ranging from 10⁻¹⁰ to 3.0 × 10⁻⁵ μM in a cell proliferation assay. Under these conditions, trioxifene inhibited PAIII cell proliferation (IC50 = 4.6 μM). The positive reference SERM, LY117018 (11), was less active than trioxifene for inhibiting PAIII cellular proliferation (IC50 = 14.7 μM; Fig. 5). In vivo administration of trioxifene failed to produce cytoreductive effects against primary PAIII tumor growth in the tail (Table 1).
Antimetastatic Effects in PAIII PCa Tumor-Bearing LW Rats.

The PAIII prostatic adenocarcinoma metastasizes in a reproducible, time-dependent manner through lymphatic channels from the tail to the gluteal and iliac lymph nodes and subsequently to the lungs (21). Histological evaluation of tissues from untreated PAIII-bearing LW rats revealed a time-dependent expansion of neoplastic cellular foci in the subcapsular sinuses of the lymph nodes draining the tail (21). After 28 days, the lymph nodes of untreated rats contained large masses of carcinoma cells. Although reduced in size, histological evaluation revealed that the lymph nodes trioxifene-treated PAIII-bearing LW rats were composed entirely of tumor cells and indistinguishable from vehicle-treated control tissues. Trioxifene-treated rats exhibited significant ($P < 0.05$) regression of gluteal and iliac lymph node weights relative to PAIII-bearing controls (Fig. 6). Maximum inhibition of PAIII lymphatic metastasis to the gluteal and iliac lymph nodes was 86 and 88%, respectively. In addition to suppressing lymph node metastases, trioxifene inhibited metastasis to the lungs. Lung colony numbers were significantly reduced ($P < 0.05$) by trioxifene administration. Maximum inhibition of pulmonary metastasis was 98% with a daily trioxifene dose of 40.0 mg/kg-day (Fig. 7).

Trioxifene Extends Survival in PAIII-Bearing LW Rats. In the survival study, administration of trioxifene (40.0 mg/kg-day) for 28 days produced significant ($P < 0.05$) antimetastatic activity as evidenced by reductions in gluteal and iliac lymph node weights relative to PAIII-bearing controls (Fig. 6). Maximum inhibition of PAIII lymphatic metastasis to the gluteal and iliac lymph nodes was 86 and 88%, respectively. In addition to suppressing lymph node metastases, trioxifene inhibited metastasis to the lungs. Lung colony numbers were significantly reduced ($P < 0.05$) by trioxifene administration. Maximum inhibition of pulmonary metastasis was 98% with a daily trioxifene dose of 40.0 mg/kg-day (Fig. 7).

PAIII-bearing rats administered trioxifene (40.0 mg/kg-day) for 28 days followed by vehicle treatment until death (mean survival, 50.11 ± 1.32 days) lived significantly ($P < 0.05$) longer than vehicle-treated control animals (mean survival, 41.78 ± 0.88 days; Fig. 10). Administration of trioxifene to PAIII-bearing rats from the day after PAIII cell injection until death significantly ($P < 0.05$) extended survival (mean survival, 64.44 ± 2.99 days) beyond the vehicle control and trioxifene (28 days) plus vehicle treatment groups.

In Vivo Accessory Sex Organ Regression and Testicular and Body Weight Effects. Trioxifene administration to PAIII-bearing rats for 28 days produced significant ($P < 0.05$) dose-related regression of normalized (for body weight changes) ventral prostatic weights with maximum inhibition of 76% (Table 1). Trioxifene also produced decreases in normalized seminal vesicular weights with maximum inhibition of 64% at a daily dose of 40.0 mg/kg-day. Trioxifene administration to PAIII-bearing rats produced significant ($P < 0.05$) increases in normalized (per 100 g of body weight) testicular weights (Table 1) over PAIII control levels. In these animals, the absolute testicular wet weights were not significantly changed from control values. Trioxifene administration to PAIII-bearing rats produced significant ($P < 0.05$) dose-related decreases in final-to-initial body weight ratios (Table 1). Maximum inhibition of body weight gain was 31% from nontreated controls at a daily trioxifene dose of 40.0 mg/kg-day.

DISCUSSION

The results of the present study demonstrate that the ERα-selective SERM trioxifene inhibits the in vitro cellular proliferation and in vivo metastasis of the androgen-independent PAIII metastatic PCa cell line in rats. PAIII cells express measurable levels of both the α and β isoforms of ER and are AR negative (28, 29). Despite this phenotypic difference, the PAIII model has utility for evaluating the potential role of ERα and ERβ in mediating tumor progression and metastasis. Our results with trioxifene confirm previously published findings of antimetastatic activity with LY117018 (15) and raloxifene (17) in this rat PCa preclinical model. In dimethylbenzanthracene-treated female rats, trioxifene administered in the dose range used in our PAIII studies was less effective than tamoxifen in preventing the development of mammary tumors (13). The antimetastatic activity of tamoxifen in the PAIII model is inferior to trioxifene. In contrast, the highest trioxifene dose tested in the PAIII model (40.0 mg/kg-day, or ∼240.0 mg/m²) was comparable to an active dose of the drug (200.0 mg/m²) evaluated in human breast cancer patients (30). Our data add to a growing body of evidence that suggests potential therapeutic utility of SERMs such as trioxifene in PCa patients with androgen-independent metastatic disease. A recent publication demonstrated the AR-independent activation of caspase-9-related apoptotic activity of a related SERM, raloxifene, in LNCaP cells. These data have provided part of the preclinical rationale for ongoing human clinical trials with raloxifene in PCa patients (19).

Trioxifene is an active competitor for human ERα, binding with approximately 20-fold selectivity over the ERβ isoform. The compound is antagonistic for ERα-mediated luciferase activity in PAIII cells at concentrations exceeding 100 nm. Trioxifene did not markedly alter ERβ-mediated luciferase activity at any concentration tested in these same cells. Given these in vitro responses, trioxifene can be considered a SERM with selective ERα-antagonistic properties in a malignant prostatic epithelial cellular environment. PAIII rat prostatic...
TABLE 1  Trioxifene effects on body weight gain, ventral prostatic and seminal vesicle involution, testicular weights, and primary tumor growth in the tails of male LW rats inoculated with PAIII cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Final/Initial body weight ratio</th>
<th>Tail weight (g/100 g body wt)</th>
<th>Testicular weight (g/100 g body wt)</th>
<th>Ventral prostatic weight (mg/100 g body wt)</th>
<th>Seminal vesicle weight (mg/100 g body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAIII + vehicle (8)</td>
<td>1.888 ± 0.064</td>
<td>43.69 ± 3.25 (1015.89 ± 24.77)</td>
<td>82.2 ± 3.1 (185.0 ± 4.7)</td>
<td>77.4 ± 3.0 (174.78 ± 9.2)</td>
<td></td>
</tr>
<tr>
<td>No PAIII + vehicle (9)</td>
<td>1.790 ± 0.051</td>
<td>46.73 ± 1.32 (1004.0 ± 56.97)</td>
<td>90.0 ± 4.1 (195.9 ± 9.2)</td>
<td>81.8 ± 1.8 (178.22 ± 5.7)</td>
<td></td>
</tr>
<tr>
<td>PAIII + trioxifene (mg/kg-day)</td>
<td></td>
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</tr>
<tr>
<td>2.0 (8)</td>
<td>1.459 ± 0.046</td>
<td>56.61 ± 0.58 (604.12 ± 17.61)</td>
<td>60.9 ± 2.6 (99.8 ± 5.8)</td>
<td>45.5 ± 2.7 (75.4 ± 6.0)</td>
<td></td>
</tr>
<tr>
<td>4.0 (7)</td>
<td>1.371 ± 0.037</td>
<td>54.01 ± 1.24 (934.00 ± 58.21)</td>
<td>51.9 ± 6.8 (71.9 ± 8.9)</td>
<td>39.4 ± 5.5 (55.2 ± 7.4)</td>
<td></td>
</tr>
<tr>
<td>20.0 (9)</td>
<td>1.357 ± 0.039</td>
<td>57.97 ± 2.80 (888.00 ± 21.25)</td>
<td>46.5 ± 1.1 (85.1 ± 11.0)</td>
<td>34.0 ± 4.3 (64.4 ± 8.4)</td>
<td></td>
</tr>
<tr>
<td>40.0 (10)</td>
<td>1.296 ± 0.021</td>
<td>56.01 ± 5.32 (873.25 ± 90.44)</td>
<td>29.9 ± 2.4 (46.0 ± 4.4)</td>
<td>18.7 ± 3.0 (28.9 ± 4.8)</td>
<td></td>
</tr>
</tbody>
</table>

* For experimental details, see “Materials and Methods” section.
† Number of observations in parentheses.
‡ Mean ± SE of the absolute organ weight (mg).
§ Significantly different from PAIII + vehicle-treated controls (P < 0.05) by Dunnett’s test on ranked data.
¶ Significantly different from PAIII + vehicle-treated controls (P < 0.05) by Dunnett’s test.

LY117018 and raloxifene in PAIII tumors, may be mediated by the ERα antagonistic properties of these SERMs. In a limited series of human androgen-independent and metastatic PCa specimens, Bonkhoﬀ et al. (34) demonstrated an association between increased levels of endogenous ERα and expression of the androgen-independent metastatic PCa phenotype. The antiproliferative effects seen with trioxifene in cell culture conditions were not extended in vivo to a reduction of primary PAIII tumor in the tail. The observed antimitotic effects of trioxifene may result from the action of ERα as an antagonist to processes involved with tumor metastasis (34) rather than as an inhibitor of growth of the metastatic lesions.

Although these studies lacked sufﬁcient numbers of PAIII-bearing rats to demonstrate a statistical response based on Kaplan–Meier analysis, trioxifene-induced decreases in metastatic tumor burden were sufﬁcient to signiﬁcantly extend the life span of PAIII-bearing rats. Survival is the key preclinical end point for the assessment of a potential antimitotic agent for human trials (35). These data support the contention that trioxifene treatment reduces lymphatic and pulmonary tumor burden and extends life span, producing signiﬁcant statistical and meaningful clinical responses in tumor-bearing animals. Despite the variability in untreated control absolute PAIII tumor burdens, trioxifene produced consistent antimitotic responses in

Adenocarcinoma cells express both ERα and ERβ but are AR negative, as evidenced by the Western blot immunoreactivity described here and the lack of in vivo responsiveness to castration of the tumor-bearing host (15). Our data from PAIII cell lysates correlate with the ﬁndings of Lau et al. (31), who demonstrated variable expression of ERβ and ERα in the AR-negative human PCa cell lines DU-145 and PC-3.

We have demonstrated an approximate 50-fold difference in trioxifene concentrations needed to compete for ERα binding and ERα-mediated luciferase gene activation as well as the antiproliferative effects of the compound in PAIII cell cultures. This differential potency between the biochemical and PAIII cellular responses may reﬂect the cellular trafﬁcking of trioxifene or the potential for this agent and other SERMs to impinge on off-target-mediated cellular pathways at concentrations in excess of levels relevant to their binding to the two ER gene products. ER-independent high-aﬃnity binding sites for SERM binding have been demonstrated in a variety of models (32, 33); however, the role of these binding proteins in mediating estrogenic and SERM cellular responses is poorly understood.

Trioxifene treatment signiﬁcantly inhibited PAIII metastasis through lymphatic channels and reduced the spread of the tumor to the lungs. One mechanism for the antimitotic action of trioxifene, like

**FIG. 6.** Inhibitory effects of trioxifene on the gluteal and iliac lymph nodal metastasis of the PAIII prostatic adenocarcinoma in male LW rats. LW rats received s.c. injections of 10⁶ PAIII cells in the tail, and trioxifene (0–40.0 mg/kg-day) was administered by the s.c. route for 28 days beginning the day after tumor cell inoculation. Gluteal and iliac lymph nodes were dissected at necropsy and weighed. *, significantly different from PAIII + vehicle; †P < 0.05) by Dunnett’s test on ranked data. Numbers of observations are listed in parentheses. Bars, SE. For details, see “Materials and Methods” section.

**FIG. 7.** Inhibitory effects of trioxifene on metastasis of the PAIII prostatic adenocarcinoma to the lungs of male LW rats. LW rats received s.c. injections of 10⁶ PAIII cells in the tail, and trioxifene (0–40.0 mg/kg-day) was administered by the s.c. route for 28 days beginning the day after tumor cell inoculation. Tumor foci were counted on the pleural surface of Bouin’s±ixed lungs removed at necropsy. *, signiﬁcantly different from PAIII + vehicle (P < 0.05) by Dunnett’s test on ranked data. Numbers of observations are listed in parentheses. Bars, SE. For details, see “Materials and Methods.”
The evidence is that there were no significant differences in tumor cell inoculation unless tumor cell inoculation.

Tumor foci were counted on the pleural surface of Bouin's fixed lungs removed at necropsy. *, significantly different from PAIII + vehicle (P < 0.05) by Dunnett's test on ranked data. Numbers of observations are listed in parentheses. Bars, SE. For details, see "Materials and Methods."

Fig. 8. Inhibitory effects of trioxifene (40.0 mg/kg-day; 28-day interim analysis of survival study) on the gluteal and iliac lymph nodal metastasis of the PAIII prostatic adenocarcinoma in male LW rats. LW rats received s.c. injections of 10^6 PAIII cells in the tail, and trioxifene (40.0 mg/kg-day) or vehicle was administered by the s.c. route for 28 days following the day after tumor cell inoculation. Gluteal and iliac lymph nodes were dissected at necropsy and weighed. *, significantly different from PAIII + vehicle (P < 0.05) by Dunnett's test on ranked data. Numbers of observations are listed in parentheses. Bars, SE. For details, see "Materials and Methods."

Fig. 9. Inhibitory effects of trioxifene (40.0 mg/kg-day; 28-day interim analysis of survival study) on the metastasis of the PAIII prostatic adenocarcinoma to the lungs of male LW rats. LW rats received s.c. injections of 10^6 PAIII cells s.c. in the tail, and trioxifene (40.0 mg/kg-day) or vehicle was administered by the s.c. route for 28 days following the day after tumor cell inoculation. Tumor foci were counted on the pleural surface of Bouin's fixed lungs removed at necropsy. *, significantly different from PAIII + vehicle (P < 0.05) by Dunnett's test on ranked data. Numbers of observations are listed in parentheses. Bars, SE. For details, see "Materials and Methods."

Fig. 10. Extended survival of PAIII-bearing male LW rats administered trioxifene (40.0 mg/kg-day for 28 days or continuously until death). LW rats received s.c. injections of 10^6 PAIII cells in the tail, and trioxifene (40.0 mg/kg-day) or vehicle was administered by the s.c. route following the day after tumor cell inoculation. Animals were treated with trioxifene for 28 days or continuously until death. *, significantly different from PAIII + vehicle (P < 0.05); #, significantly different from PAIII + trioxifene (28 days; P < 0.05) by Dunnett's test. Number of observations are listed in parentheses. Bars, SE. For details, see "Materials and Methods."

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the metastatic processes in the rat PAIII model may be similar in human PCs, trioxifene deserves consideration for clinical evaluation in humans for this indication. In addition, understanding the anti metastatic mechanism(s) of trioxifene action in PCs may provide new approaches to the treatment of androgen-independent metastatic disease.

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