Effect of Steroidal and Nonsteroidal Antiestrogens on the Growth of a Tamoxifen-stimulated Human Endometrial Carcinoma (EnCa101) in Athymic Mice

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ABSTRACT

Tamoxifen (TAM), a nonsteroidal antiestrogen, is used in the adjuvant treatment of breast cancer. Previous studies, however, have indicated that some human breast and endometrial tumors are stimulated to grow with TAM in the athymic mouse. One such TAM-stimulated tumor is the EnCa101 human endometrial adenocarcinoma. Our aim was to evaluate the ability of different doses of TAM or other nonsteroidal antiestrogens to stimulate the growth of EnCa101 tumors in athymic mice. Additionally, we have evaluated less estrogenic antiestrogens (two steroidal antiestrogens, RU 39,411 and ICI 164,384, and two nonsteroidal antiestrogens, keoxifene and MER-25) for their ability to inhibit TAM-stimulated growth. All experiments were done in ovariectomized athymic mice transplanted in the axillary mammary fat with 1-mm³ pieces of EnCa101 tumor. Sustained release preparations (0.5-2.0-cm Silastic capsule or 5-mg TAM cholesterol pellet) of TAM caused similar tumor growth. The growth rate was not altered by an additional daily i.p. injection of 1 mg TAM in 0.1 ml peanut oil. A 3-mg TAM daily dose was toxic. Four weeks of treatment (100-μg s.c. injections, every other day) with nonsteroidal antiestrogens, trioxifene mesylate, enclomiphene, or nafoxidine, stimulated tumor growth. However, keoxifene stimulated this tumor to a lesser degree than TAM and partially inhibited TAM-stimulated growth. ICI 164,384 showed no stimulatory activity (1-mg s.c. injections every other day) alone compared to controls but inhibited TAM-stimulated (0.25-cm Silastic capsule) growth. In a parallel experiment, RU 39,411 (1-mg s.c. injections every other day) stimulated EnCa101 to grow. In contrast when RU 39,411 was administered in a sustained release preparation (2.0-cm Silastic capsule) there was no stimulatory growth compared to controls. Additionally RU 39,411 inhibited TAM-stimulated growth, but the low-potency antiestrogen, MER-25, was less effective in this regard. These data suggest that less "estrogenic" antiestrogens can inhibit TAM-stimulated tumor growth in vivo. Thus these compounds or derivatives may prove useful as a second-line endocrine therapy should TAM-stimulated tumor growth occur in the clinic.

INTRODUCTION

TAM is a nonsteroidal antiestrogen that has been used successfully in the long-term treatment of endocrine-dependent breast cancer (1-4). In addition, TAM has been shown to have some success in the treatment of endometrial carcinoma (5-8). However, a recent clinical report by Fornander et al. (9) has demonstrated an increase in endometrial carcinoma during breast cancer (1-4). In addition, TAM has been shown to have some success in the treatment of endometrial carcinoma (5-8). However, a recent clinical report by Fornander et al. (9) has demonstrated an increase in endometrial carcinoma during the long-term treatment of endocrine-dependent breast cancer (1-4). In addition, TAM has been shown to have some success in the treatment of endometrial carcinoma (5-8). However, a recent clinical report by Fornander et al. (9) has demonstrated an increase in endometrial carcinoma during the long-term treatment of endocrine-dependent breast cancer (1-4). In addition, TAM has been shown to have some success in the treatment of endometrial carcinoma (5-8). However, a recent clinical report by Fornander et al. (9) has demonstrated an increase in endometrial carcinoma during the long-term treatment of endocrine-dependent breast cancer. We have reported cases of human endometrial carcinoma and breast carcinomas that are stimulated to grow with TAM in athymic mice (9-11). This estrogenic effect does not seem to be a result of the estrogenic pharmacodynamics of TAM in the mouse (12, 13). Rather, both estrogenic and antiestrogenic actions of TAM can be seen in the same mouse host; TAM is stimulatory for EnCa101 human endometrial tumors while it is inhibitory for cotransplanted MCF-7 wild-type breast tumors (14).

The aim in this paper was to establish whether the EnCa101 endometrial carcinoma is stimulated to grow in vivo with structurally related nonsteroidal antiestrogens and to determine whether novel less estrogenic steroidal antiestrogens (RU 39,411 and ICI 164,384; Fig. 1) could inhibit the TAM-stimulated growth.

MATERIALS AND METHODS

Tumors. EnCa101 tumors were continuously passaged in athymic mice with 5-mg TAM pellets as described previously (11). Tumors were transplanted into 4-5-week-old BALB/c ovariectomized athymic mice (Harlan Sprague Dawley, Indianapolis, IN). Mice were housed in laminar flow hoods with sterile cages and bedding. Tumors were measured using vernier calipers at weekly intervals and the mean cross-sectional area was calculated as

\[
\text{Length} \times \text{Width} \times \pi \times 1/2
\]

Drug Therapies. Compounds used in the experiments (Fig.1) were gifts from the following sources: TAM free base, ICI Pharma, Wilmington, DE; RU 39,411 and ICI 164,384, Roussel Uclaf, Romainville, France; keoxifene (LY 156,758) and trioxifene mesylate (LY 133,314), Lilly Research Laboratory, Indianapolis, IN; enclomiphene and MER-25, Merrill Dow Laboratories, Cincinnati, OH; nafoxidine, Upjohn Laboratories, Kalamazoo, MI

The experiments were conducted over a 2-year period with different drug delivery systems. The aim was to avoid any drug contact by using sustained-release preparations or, where necessary, to ensure comparable drug administration.

 Different methods were used for the administration of TAM during the experimental period. TAM-free base (5 mg, 4-week release) cholesterol pellets were custom made by Innovative Research of America, Toledo, OH. Silastic capsules used in the experiments were made from Silastic tubing (Dow Corning, Midland, MI) 0.125 inch outside diameter x 0.078 inch inside diameter. This was cut to various lengths (0.25-2.0 cm) and packed with TAM. The ends were plugged with Silastic cement (Dow Corning). All capsules were sterilized by γ-irradiation. In experiments in which TAM was injected either i.p. or s.c., the compound was weighed, dissolved in 100% ethanol, and mixed with peanut oil. The ethanol was evaporated under a gentle stream of nitrogen while mixing at 45° C.

Other antiestrogens were administered in similar ways. Keoxifene was made into cholesterol pellets containing 5 mg by Innovative Research of America. Various antiestrogens were injected s.c. as 100 μg/0.1 ml peanut oil 3 times weekly. The antiestrogens RU 39,411 or MER-25 were administered as 2-cm Silastic capsules or the antiestrogens RU 39,411 and ICI 164,384 were injected s.c. every other day at a concentration of 1 mg/0.1 ml peanut oil as a fine suspension. Preliminary experiments showed that ICI 164,384 was not released from Silastic capsules.

Stimulatory Activity of Antiestrogens. The comparability of different methods of administration of TAM was determined. Animals with implanted tumors were also implanted with 0.25, 1.0-, 2.0-, or 2.0-cm Silastic capsules of TAM, 5-mg cholesterol pellets (changed every 4 weeks) or, in a separate experiment, 2.0-cm capsules of TAM were implanted, and 1 or 3 mg TAM daily (5 days/week) were administered by i.p. injection. To study the ability of various antiestrogens (TAM)}
INHIBITION OF ENDOMETRIAL CARCINOMA GROWTH

Fig. 1. Chemical structures of the antiestrogens tamoxifen, enclomiphene, nafoxidine, trioxifene, MER-25, keoxifene, RU 39,411, and ICI 164,384.

Tamoxifen, enclomiphene, nafoxidine, trioxifene, MER-25, keoxifene, trioxifene, enclomiphene, and nafoxidine) to promote the growth of EnCa101, animals were given injections 3 times weekly (Monday, Wednesday, Friday) with 100 µg s.c.

Inhibitory Activity of Antiestrogens. Several experiments were conducted to inhibit TAM-stimulated growth of EnCa101. Keoxifene (5 mg cholesterol pellet) was coadministered with TAM (5-mg cholesterol pellet) to determine the inhibitory activity of the compound. Similarly, Silastic capsules (2 cm) of RU 39,411 or MER-25 were implanted into tumor-bearing mice to determine whether these compounds would inhibit growth stimulated by TAM (5-mg cholesterol pellet). Finally, RU 39,411 was compared with ICI 164,384 (1-mg suspension in 0.1 ml peanut oil s.c. every other day) to inhibit TAM (0.25-cm Silastic capsule) stimulated tumor growth. The injection method of administration of the steroidal antiestrogens was used because ICI 164,384 cannot be administered by Silastic capsule.

Statistical Analysis. Differences in mean tumor area were measured using analysis of variance followed by unpaired Student’s t test.

RESULTS

Stimulatory Activity of Antiestrogens. During these experiments, different delivery systems were used to administer TAM. All early studies used cholesterol pellets exclusively and this was a standard method for the sustained-release administration of TAM (11). The delivery system for TAM provides enough drug to inhibit estradiol-stimulated growth of MCF-7 breast cancer cells implanted into athymic mice (11). We have subsequently used Silastic capsules of TAM to deliver the drug. Capsules (0.25, 1.25, and 2.5 cm) will inhibit estradiol-stimulated growth of MCF-7 breast tumors and ovariectomized athymic mouse uterine wet weight (15). In the current study, TAM capsules (0.25, 1.0, and 2.0 cm) produced a similar growth rate of implanted EnCa101 and this in turn was similar to the growth rate of the TAM-cholesterol pellet. These data are illustrated in Fig. 2 (2-cm Silastic capsule). Fig. 4 (TAM-cholesterol pellet) and Fig. 6 (0.25-cm Silastic capsule) demonstrate comparable growth curves with TAM.

We wanted to ensure that large doses of TAM would not be completely inhibitory to the growth of EnCa101. Animals with tumors were implanted with 2.0-cm TAM Silastic capsules and given daily i.p. injections (5 days/week) of either vehicle (peanut oil) or vehicle containing 1 or 3 mg TAM. The additional 1 mg TAM daily did not affect the growth rate of EnCa101 (Fig. 2B); however, 3 mg TAM daily was toxic and all animals died by the end of the third week of treatment.

We determined whether nonsteroidal antiestrogens other than TAM could stimulate the growth of EnCa101 tumor in athymic mice. Animals implanted with tumors were given 3 times weekly injections (s.c.) of 100 µg of each antiestrogen, and tumor size was measured for 4 weeks (Fig. 3). Control tumors in this experiment had a tumor growth of 0.18 ± 0.02 (SE) cm² which was significantly lower than the TAM-stimulated group (P < 0.001) at 0.76 ± 0.4 cm².

Trioxifene, enclomiphene, nafoxidine, and keoxifene all stimulated the growth of EnCa101 compared with controls. Triox-
ifene had the largest mean tumor area (0.92 ± 0.12 cm²) of all five compounds tested; however, the mean tumor area of trioxifen, enclomiphene, and nafoxidine groups did not differ significantly (P > 0.05) from TAM-treated animals at the same doses. In contrast, keoxifene produced a lower tumor area 0.58 ± 0.05 cm² which was significantly different from that of the TAM group (P < 0.02). Since keoxifene appeared to be less stimulatory than TAM, we tested the ability of keoxifene to inhibit the TAM-stimulated growth of EnCa101 tumors.

Inhibitory Activity of Antiestrogens. Mice were transplanted with EnCa101 tumors and treated with 5-mg TAM and 5-mg keoxifene pellets (replaced every 4 weeks) alone or with both pellets together. After 6 weeks of observation (Fig. 4), TAM-stimulated growth of EnCa101 tumors had achieved a mean size of 1.57 ± 0.06 cm² which was significantly (P < 0.001) larger than that of controls (0.42 ± 0.01 cm²). Interestingly, keoxifene at the same dose had a mean tumor area of 0.78 ± 0.03 cm² which was significantly lower than that of the TAM-treated group (P < 0.001) and this was almost identical to the mean tumor area (0.80 ± 0.05 cm²) of animals treated with TAM and keoxifene. Thus, keoxifene is less effective at stimulating EnCa101 tumor growth than TAM in the athymic mouse and inhibits TAM-stimulated tumor growth.

Using the same dose of TAM (5-mg pellet), we studied the ability of RU 39,411, a steroidal antiestrogen, and MER-25, a nonsteroidal antiestrogen, to inhibit TAM-stimulated growth. Both compounds are less estrogenic than TAM. After 6 weeks of treatment (Fig. 5), TAM-treated animals had a tumor area of 1.81 ± 0.03 cm² compared to only 0.26 ± 0.01 cm² for placebo-treated animals (P < 0.001). Preliminary studies using custom-made 5-mg pellets of RU 39,411 demonstrated that these were irritating to the skin and therefore this method could not be used as a delivery system. The RU 39,411 and MER-25 administered s.c. in a 2.0-cm Silastic capsule did not show any significant increase in mean tumor area over that in placebo-treated animals (data not shown). However, the tumors treated with RU 39,411 capsules and 5-mg TAM pellets had a mean tumor area of 0.51 ± 0.04 cm² which was significantly different from that of the TAM-treated group (p<0.001). Animals treated with MER-25 capsules and TAM pellets also had a significantly lower mean tumor area (1.45 ± 0.16) than TAM-treated only tumors (P < 0.02). Thus, although RU 39,411 did not inhibit tumor area to placebo levels, it was apparently more active than keoxifene or MER-25 and had less stimulatory activity than keoxifene on tumor size than any of the compounds tested previously. MER-25, although it could partially inhibit TAM-stimulated growth, was less effective than RU 39,411 using the Silastic capsule delivery system.

Finally, we tested the effects of ICI 164,384 on TAM-stimulated growth of EnCa101 tumors in athymic mice and compared this activity to RU 39,411 in a parallel experiment (Fig. 6). For this experiment, we used 0.25-cm TAM Silastic capsules instead of 5-mg pellets. The rationale was to use a minimal amount of TAM to cause maximal stimulation of EnCa101 growth similar to that seen with 5-mg TAM pellets. Both nonsteroidal antiestrogens were injected every 48 h s.c. at a dose of 1 mg/0.1 ml in the form of an aqueous fine suspension (ICI 164,384 could not be administered in Silastic capsules).

In contrast to the previous experiment (Fig. 5), RU 39,411 alone was significantly stimulatory (P < 0.01) compared with control animals. In addition, animals treated with a sustained-release, 0.25-cm TAM Silastic capsules and RU 39,411 showed no decrease in mean tumor area compared to TAM-only treated animals (Fig. 6A). ICI 164,384 significantly inhibited (P < 0.01) the growth of TAM-stimulated EnCa101 tumor (Fig. 6B). After 6 weeks of treatment, the tumor area of the TAM-treated group was 1.42 ± 0.02 cm² compared with a TAM ± ICI 164,384-treated group of 0.72 ± 0.03 cm². ICI 164,384-treated animals alone showed no sustained tumor growth compared with the control animals.

DISCUSSION

These experiments describe the stimulation of EnCa101 tumors in athymic mice by a range of nonsteroidal and steroidal...
antiestrogens. EnCa101 human endometrial carcinoma has previously been shown to be stimulated to grow in athymic mice with TAM (14, 16). Paradoxically TAM has been used with some success in the clinic for the treatment of endometrial carcinoma (5-7). Nevertheless, failure with antiestrogen therapy eventually occurs in the clinic with TAM whenever it is used in either endometrial or breast cancer (1-4, 7).

Antiestrogens have a diverse pharmacology in different species such as mice, rats, and humans (17). In the mouse, for example, TAM is considered to be a full estrogen in the uterus, keoxifene has partial estrogenic characteristics, and MER-25 has no estrogenic characteristics in this species (17, 18). In the rat keoxifene and TAM have considerably less estrogenic action in the uterus. Nevertheless, keoxifene was less effective than TAM (at equivalent doses) in preventing tumor promotion in the N-methylnitrosurea-induced rat mammary carcinoma model (19). Consequently, the characteristics of an antiestrogen can change depending on the end point and species in which it is studied. We, therefore, believed it was important to evaluate whether other antiestrogens related to TAM would support the growth of EnCa101 in athymic mice. Our aim was to determine whether all compounds that are considered to exert their actions through the estrogen receptor (17) will cause growth of EnCa101 or whether compounds could be found that would block TAM-stimulated growth. The nonsteroidal antiestrogens, keoxifene and MER-25, were selected because they are known to exhibit less estrogenic properties than TAM (20, 21).

Initially we compared all of the nonsteroidal antiestrogens (which were available to us at the time) at the same dose to establish their ability to stimulate the growth of the EnCa101 tumor. After 4 weeks of treatment, only keoxifene had a mean tumor area which was significantly less than (P < 0.03) the TAM-treated group (Fig. 3). Furthermore keoxifene inhibited TAM-stimulated growth of EnCa101 tumors when given simultaneously. The finding that keoxifene had inhibitory activity in this tumor system encouraged us to test two new steroidal antiestrogens, RU 39,411 and ICI 164,384, and the nonsteroidal antiestrogen, MER-25, for their ability to stimulate and/or inhibit TAM-stimulated growth of EnCa101 tumors. When RU 39,411 was compared to TAM and administered in a sustained-release preparation, this compound was better than keoxifene and MER-25 at inhibiting TAM-stimulated growth. However, in a separate experiment (Fig. 5A) when RU 39,411 was injected s.c., 1 mg every other day, there was a stimulation of EnCa101 growth that was comparable to TAM stimulation. These conflicting results may reflect differences in the mode of administration (s.c. injection or s.c. sustained release Silastic capsule) of the compound. It has been our experience that RU 39,411 inhibits the TAM-stimulated growth of a variant MCF-7 tumor grown in athymic mice (MCF-7 TAM) at the identical injectable doses (22). Therefore, the disparate actions of this antiestrogen may be due to tissue specificity as seen previously with TAM.

Finally parallel studies done with ICI 164,384 confirm results attained with TAM-stimulated breast tumors (22). This antiestrogen did not stimulate tumor growth in EnCa101 tumors or MCF-7 TAM tumors and also appears to inhibit TAM-stimulated growth effectively in both models. We have repeated the experiment with ICI 164,384 and confirmed our results. This report supports data by Wakeling and Bowler (23) that the estrogenic activity of ICI 164,384 is negligible.

In conclusion, we have demonstrated differing stimulatory and inhibitory activities for antiestrogens on the growth of the EnCa101 tumor in the athymic mouse. Since TAM-stimulated tumors may represent a form of antiestrogen resistance, these results demonstrate that new antiestrogens may be useful in the clinic as a second-line endocrine therapy for patients failing TAM.

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