Effect on Growth and Cell Cycle Kinetics of Estradiol and Tamoxifen on MCF-7 Human Breast Cancer Cells Grown in Vitro and in Nude Mice

Nils Brünnert, Diane Bronzert, Lars L. Vindeløv, Kaare Rygaard, Mogens Spang-Thomsen, and Marc E. Lippman

ABSTRACT

The effects of estradiol and tamoxifen (TAM) on the estrogen-dependent human breast cancer cell line MCF-7 grown in vitro and in nude mice were compared. The effect on growth was determined by cell number in vitro and by tumor growth curves in nude mice. The effects on the cell cycle kinetics were determined by repeated flow cytometric DNA analyses in vitro and in vivo and by the technique of labeled mitosis in nude mouse-grown tumors.

Under in vitro conditions, estradiol induced a pronounced increase in S-phase fraction and cell number. TAM inhibited growth of MCF-7 cells with a concomitant increase in the G1 phase from 60% to 75%. In nude mice, MCF-7 only formed tumors in estradiol-supplemented mice. No differences were observed in growth and cell kinetics between 0.1 and 1.0 mg of estradiol. Daily i.p. injections of TAM resulted in tumor growth inhibition with shrinkage of tumors. The flow cytometric DNA analysis and percentage of labeled mitosis investigations revealed no significant differences in the proliferation kinetics of TAM-treated and control tumors. Calculating the cell loss factor demonstrated an increase from 69% in control tumors to 107% in TAM-treated tumors.

These experiments have shown that the cell kinetic effect of TAM is different when MCF-7 cells are grown in vitro versus in vivo. In contrast to the in vitro data, the in vivo data indicate that the growth-inhibitory effect of TAM is not mediated through a perturbation of the cell cycle.

INTRODUCTION

It has recently been shown that TAM exposure of estrogen receptor-positive human breast cancer cell lines grown in vitro inhibits cell proliferation by a mechanism involving an accumulation of cells in the G1 phase of the cell cycle (1, 2). Subsequent estradiol administration releases the cells from the G1 block, resulting in a cohort of cells entering the S phase. Using the MCF-7 human breast cancer cell line, Weichselbaum et al. (3) demonstrated that an estradiol-induced increase in S-phase cells enhanced the sensitivity of the culture to a subsequent applied S-phase-specific cytotoxic drug.

The combination of endocrine-manipulative and chemotherapeutic regimens has been widely used hoping for further improvements in the management of breast cancer. However, the improvement in patient survival has not been convincing. In order to increase the clinical outcome of combining endocrine- and chemotherapy, clinical studies have now been initiated utilizing the cell cycle changes described for estradiol and TAM treatment of breast cancer cells grown in vitro (4-6).

To date, little information has been available on the cell kinetic effect of estradiol and TAM on human breast cancer in vivo. Recently, the effects of estradiol as well as of TAM on the MCF-7 human breast cancer grown in nude mice were reported (7, 8). It was found that estradiol-induced growth inhibition was accompanied by a decrease in the fraction of cells in G1 with the subsequent formation of polyploid cells. Following TAM treatment no significant changes were observed between the cell cycle distributions of TAM-treated and control tumors as measured by FCM, although the treatment significantly inhibited the growth of the tumor. Osborne et al. (9) reported on the effect of TAM on the mitotic index of the MCF-7 breast cancer grown in nude mice. They were not able to demonstrate any significant differences between treated and control tumors, although the tumor growth was inhibited by the drug.

Because of the obvious inconsistency between the cell kinetic results obtained following TAM treatment of in vitro and in vivo grown human breast cancer cells, we have compared the in vitro effect of estradiol and TAM in the MCF-7 breast cancer cell line with the effect of this drug when the same breast cancer cell line was grown as solid tumors in the nude mouse.

We report here that, in contrast to in vitro conditions, TAM had no cell cycle kinetic effect in vivo as determined by FCM and PLM, although the drug significantly inhibited tumor growth. Calculating the cell loss factor revealed an increase from 69% in control tumors to 107% in TAM-treated tumors.

MATERIALS AND METHODS

In Vitro

Cells and Tissue Culture. The MCF-7 cell line (10) was a gift from Dr. Marvin Rich, Michigan Cancer Foundation (Detroit, MI). MCF-7 cells were passaged in improved minimal essential medium as modified by Richter and coworkers (11) (Grand Island Biological Co., Grand Island, NY), supplemented with gentamycin (40 mg/liter) and 10% fetal calf serum (Grand Island Biological Co.).

Cell Proliferation Experiments. In vitro MCF-7 growth experiments were done as previously described (12). In brief, the cells were plated at approximately 100,000 cells/35-mm well in improved minimal essential medium supplemented with 5% charcoal-stripped calf serum. The following day, 17β-estradiol (10^-8 M) (Sigma Chemical Co.) or TAM (10^-7 M) obtained from ICI America, Inc. (Wilmington, DE), was added from 1000-fold ethanol stocks. Controls contained 0.1% ethanol carrier. Cells were harvested at different time points after hormone exposure, and an aliquot was removed for cell counting. The remainder was centrifuged at 800 × g, and the cell pellet was resuspended in a citrate buffer with dimethyl sulfoxide and immediately stored at -80°C until analyzed. Storage of samples, preparation, and staining by propidium iodide were performed as described elsewhere (13-15). The flow cytometer used was a FACS III cell sorter (Becton Dickinson, Sunnyvale, CA). The DNA distributions were analyzed with a computer program calculating the fraction of cells in the G1, S, and G2 + M phases (16).

In Vivo

Tumors and Nude Mice. MCF-7 cells were transferred from in vitro conditions into 6-wk-old estradiol-supplemented intact female nude mice (NMRI) by s.c. inoculation of 0.1 ml of culture medium containing approximately 2 x 10^6 cells into the flanks of the recipient mice. Growing tumors were serially passaged by s.c. transplantation of 2-mm-diameter tumor blocks under general anesthesia. The mice were kept under sterile conditions and were given sterile food and water ad
In the present experiments MCF-7 tumors in their seventh passage in nude mice were used.

Growth Curves. Tumors were measured in two dimensions ($d_1$ and $d_2$) 3 times a week using a sliding gauge, and the tumor area, $A = d_1 \times d_2$, was calculated. The $A$ values were used to construct mean normalized transformed Gompertz growth curves according to Ref. 17

$$\ln[\ln(A_{\text{max}}) - \ln(A(t))] = \ln(\beta/a) - a t,$$

where $A(t)$ is the tumor area at time $t$, $A_{\text{max}}$ is the theoretical maximum area, and $\alpha$ and $\beta$ are constants. The $\ln(A_{\text{max}})$ was graphically estimated to be $T_0$ for the MCF-7 tumor (17). In this equation the tumor size at time $t$ is subtracted from the estimated maximum area.

In growing tumors this results in a negative slope (\(\alpha\)) of the growth curve, and in regressing tumors in a positive slope. Tumor volume was calculated from tumor area as described elsewhere (17). Eleven, 12, and 9 tumors were included in the 1.0-mg estradiol, 0.1-mg estradiol, and TAM groups, respectively. Furthermore, to ensure the growth-inhibitory effect of TAM in the PLM investigation, growth recordings of an additional number of 6 control and 6 TAM-treated tumors were included in the PLM investigation.

Hormone Treatment. At the day of transplantation, the mice received either 0.01 mg, 0.1 mg, or 1.0 mg of estradiol (Progynon Depot, Schering AG) as a single injection into the thigh. TAM treatment was given as daily injections of 0.1 mg of TAM. TAM was a gift from ICI, England. TAM was made up fresh daily by adding 0.52 mg of citric acid, 0.25 ml of ethanol, 0.15 ml of Tween 80, and 3.60 ml of 0.9% NaCl to 2 mg of TAM base. Control animals received the solvent. The TAM treatment was initiated on Day 32 and terminated on Day 56 after transplantation.

Cell Kinetic Studies. Tumor tissue for FCM was obtained by fine needle aspiration 3 times a week starting at the day of TAM treatment. Individual tumors were aspirated with intervals of at least 3 days to avoid admixture to the samples of aspiration-induced hemorrhage (7). Three tumors from each of the groups were aspirated at each time point. The tumors included in the FCM studies were the same as those included in the growth curve experiment.

The PLM investigations were performed in a separate experiment. PLM was performed on control tumors (mice supplemented with 0.1 or 1.0 mg of estradiol; 17) from Day 36 after transplantation and in TAM-treated tumors (0.1 mg of TAM) at Day 8 after initiation of daily TAM treatment. The pulse labeling with tritiated thymidine and preparation of automicroradiographs were performed as previously described (18).

At least 100 labeled and unlabeled mitoses were scored in representative areas of each tumor, and the PLM (labeled/total mitoses x 100) was calculated. Mitoses labeled by four or more grains were considered positive. Furthermore, the LI was determined by counting of at least 100 labeled mitoses in automicroradiographs from tumors excised 1 h after labeling.

Total numbers of 49 and 23 tumors were used in the PLM investigations of estradiol-treated and TAM-treated tumors, respectively. The PLM data were analyzed by a computer program (19), calculating the duration of the postmitotic phase ($T_{p_2}$), the DNA synthesis phase ($T_s$), the premitotic phase ($T_{p_1}$), and the median cell generation time ($T_c$), together with the frequency distribution of $T_c$. In addition, FCM was performed on 11 tumors from each of the three groups (0.1 mg of estradiol, 1.0 mg of estradiol, and 0.1 mg of TAM) on the day of initiation of the PLM investigations.

Derived Parameters. Growth curve parameters were used to calculate the tumor volume doubling time (20).

$$T_0 = -1/\alpha \ln[1 + \ln(2/[\ln(\alpha t)/\ln(A_{\text{max}})])]$$

which was derived from the Gompertz growth function (17). The potential doubling time ($T_{p_{\text{opt}}}$) was calculated from Ref. 20.

$$T_{p_{\text{opt}}} = \lambda T_d/LI$$

and

$$\lambda = T_{p_{\text{opt}}}/T_d \left[\exp([\ln(2/T_{p_{\text{opt}}})](T_{c_1} + T_{c_2})) - \exp([\ln(2/T_{p_{\text{opt}}})T_{c_2}])\right]$$

In the calculations of $T_{p_{\text{opt}}}$, the thymidine LI was used. From the values of $T_d$ and $T_{p_{\text{opt}}}$, the cell loss factor ($\Phi$) was calculated (20).

$$\Phi = 1 - T_{p_{\text{opt}}}/T_d$$

The potential doubling time and the computed cell generation time ($T_c$) were applied for the calculation of the growth fraction (20)

$$\ln a = (T_c/T_{p_{\text{opt}}}) \ln 2$$

and

$$GF = a - 1$$

RESULTS

In Vitro. MCF-7 cells grew well with a doubling time of about 24 h. The growth of the cells was stimulated significantly by estradiol and inhibited by TAM (not shown). The FCM analysis showed that estradiol decreased the fraction of cells in G1 from 60% to approximately 40% with a concomitant increase of cells in S from 27% to 40% during the first 24 h. Estradiol-treated cells reached confluence before control cells due to the stimulation by estradiol, and when confluence the G1 phases went up. TAM increased the fraction of cells in the G1 phase from control levels of about 60% to approximately 75% at 24 h after TAM exposure.

In Vivo. Different doses of estradiol were used to find the lowest concentration of estradiol needed to provide tumor growth throughout the experimental period. Following the 1.0-mg estradiol dose, tumors grew rapidly, but no growth inhibition was observed following subsequent TAM treatment. Lowering the dose of estradiol to 0.1 mg resulted in tumor growth with a similar $\alpha$ value as observed in the 1.0-mg estradiol group (Table 1). With the 0.1-mg dose, significant growth inhibition

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>T0 (days)</th>
<th>Tc1 (days)</th>
<th>Ts (days)</th>
<th>Tc2 (days)</th>
<th>LI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mg</td>
<td>26.0 ± 23.1</td>
<td>20.5 ± 11.7</td>
<td>11.7 ± 3.8</td>
<td>14.7 ± 4.0</td>
<td>31.2 ± 2.1</td>
</tr>
<tr>
<td>0.01 mg</td>
<td>43.0 ± 31.2</td>
<td>34.0 ± 23.4</td>
<td>26.5 ± 11.7</td>
<td>16.3 ± 3.8</td>
<td>31.2 ± 2.1</td>
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</tbody>
</table>

Flow cytometric DNA analyses

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<tr>
<th>Phase</th>
<th>G1</th>
<th>S</th>
<th>G2 + M</th>
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<tr>
<td>1.0 mg estrogen</td>
<td>75.3 ± 4.7</td>
<td>17.6 ± 4.0</td>
<td>4.1 ± 1.5</td>
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<tr>
<td>0.1 mg estradiol</td>
<td>82.8 ± 6.6</td>
<td>13.6 ± 5.8</td>
<td>3.6 ± 1.5</td>
</tr>
<tr>
<td>TAM</td>
<td>78.5 ± 6.4</td>
<td>16.7 ± 6.0</td>
<td>4.8 ± 0.9</td>
</tr>
</tbody>
</table>

Growth curves

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>T0 (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mg estradiol</td>
<td>-0.0140</td>
<td>12.550</td>
</tr>
<tr>
<td>0.01 mg estradiol</td>
<td>-0.0157</td>
<td>12.550</td>
</tr>
<tr>
<td>TAM</td>
<td>0.0040</td>
<td>12.550</td>
</tr>
</tbody>
</table>

* Computed median cell generation time ($T_c$) and mean times of $T_{c_1}$, $T_{s}$, and $T_{c_2}$ phases of the cell cycle (h).

* LI, [3H]thymidine labeling index.

* Mean ± SD.

* The tumor volume doubling time $T_d$, the proportionality constant $\alpha$, the potential doubling time $T_{p_{\text{opt}}}$ the cell loss factor $\Phi$, the number of proliferating cells produced per division $\alpha$, and the growth fraction (GF) were calculated as described in "Materials and Methods."
Table 1. Cell kinetic parameters from all three groups are given in control tumors (Fig. 2A) was even smaller in treated tumors of tamoxifen-treated tumors (D); posttherapeutic growth of control tumors (A); posttherapeutic growth and 9 tamoxifen-treated tumors were included. Pretherapeutic growth of all estradiol-supplemented female nude mice and treated with 0.1 mg of tamoxifen

The computerized PLM curves together with the frequency distribution of Tc from the 0.1-mg estradiol and the TAM group are shown in Fig. 3, A and B. The measured and derived cell kinetic parameters from all three groups are given in Table 1.

Heterogeneity in cell cycle times as indicated by large SD values and broad Tc frequency distribution curves is observed in all three groups. The PLM data from TAM-treated tumors
demonstrated enhancement; i.e., the points late in the experiment are above the computer fit (Fig. 3B). This is probably due to reutilization of [3H]thymidine from dead labeled cells (20, 21).

The growth kinetic parameters of the two estradiol-treated groups showed no differences. Similarly to the estradiol-treated tumors, a second peak appeared in the PLM curve of TAM-treated tumors, indicating the presence of cycling cells. Neither the cell generation time nor the duration of the individual cell cycle phases differed significantly between estradiol-treated and TAM-treated tumors.

The computerized median Tc values were 48, 43, and 34 h for TAM-treated, 1.0-mg estradiol, and 0.1-mg estradiol tumors, respectively (Table 1). Accordingly, the GFs which were calculated from Tc (and Tmot) showed an increase to 41.1% in the treated tumors compared to 25.5% in the 0.1-mg estradiol control group. The Tc changes thus apparently indicate a slight TAM-induced inhibition of the proliferative activity. In contrast, the FCM and LI data showed no significant differences found tend towards a stimulated proliferation in the TAM-treated tumors, i.e., increased fractions of cells in the S and G2 + M phases. These trends thus oppose the slight inhibition indicated by the Tc data. However, since the Tc determinations were based on the very poor computer fits.
Fig. 3. Percentage of labeled mitoses after [3H]thymidine pulse labeling of MCF-7 tumors grown in nude mice. The points are means from at least two individual countings, and the broken lines are the computed best-fit curves. The percentage of labeled mitoses curves (top) as well as the $T_c$ frequency distribution curves (bottom) are shown. Left: tumors grown in nude mice supplemented with 0.1 mg of 17β-estradiol; right: tumors grown in nude mice supplemented with 0.1 mg of 17β-estradiol and treated with 0.1 mg of tamoxifen daily from Day 32 and until included in the PLM investigation. The computed median cell generation times are indicated by the arrows.

beyond the first peaks (Fig. 3), these parameters are not considered very reliable, and in the interpretation of the results the emphasis was therefore put on the FCM and LI data (see “Discussion”). This is supported by the apparent increase in $T_c$ following an increased estradiol dose (Table 1).

From Table 1 it appears that the cell loss factor in TAM-treated tumors had increased to 107% compared to approximately 70% in control tumors.

DISCUSSION

This study has demonstrated that the cell kinetic effect of TAM treatment of the MCF-7 human breast cancer cell line is dependent on whether the cells are grown in vitro or in vivo.

The data obtained from the in vitro experiment, i.e., growth stimulation with concomitant increase in DNA synthesis by estradiol, and growth inhibition and $G_1$ accumulation by TAM, correspond to those previously reported by others (1, 2). This indicates no major differences in estradiol and TAM responsiveness between our MCF-7 cell line and the MCF-7 cell lines used in the above mentioned studies.

In the initial in vivo experiments, different concentrations of estradiol were utilized. However, in order to carry out an experiment with an estradiol antagonist, we decided to use a minimal dose of estradiol which was still capable of sustaining tumor growth during the experimental period. For example, using 1.0 mg of estradiol, tumors grew rapidly, but no effect of TAM on tumor growth was evident, probably because the serum concentration of estradiol in the mice (7) was too high to be competed by the dose of antiestrogen applied. Lowering the dose of estradiol to 0.1 mg still provided tumor growth throughout the experimental period, and with this dose the applied dose of TAM resulted in significant tumor regression. Differences in doses of estradiol and TAM may explain some of the conflicting results concerning tumor growth stabilization (tumor stasis) versus tumor regression following TAM treatment of MCF-7 in nude mice (9, 22).

It is of interest to note that 1.0 mg of estradiol had no stimulatory effect on the growth kinetics as compared with the 0.1 mg of estradiol group. Osborne et al. (9) recently demonstrated the dose-dependent effect of estradiol on MCF-7 tumors grown in nude mice. Most of the doses used in their study were below those used in the present investigation. However, when they used the highest dose of estradiol, no further growth stimulation was observed. Thus, both studies suggest an upper threshold for estradiol stimulation of MCF-7 tumors in vivo, above which no further stimulation can be obtained.

In the present in vivo experiment, TAM treatment resulted in a significant reduction in size of the tumors. The dose of TAM, 0.1 mg i.p. daily, was chosen since it has previously been shown that a single injection of 0.1 mg of TAM i.p. results in serum TAM concentrations corresponding to the therapeutic range in humans (23). TAM was administered as a daily injection for 24 days. However, since it has recently been shown that the half-life of TAM in mice is approximately 9 days (23), daily injections could probably have been avoided.

A prerequisite for investigating the cell kinetics by the PLM technique is that the cells in question are in a steady state of proliferation. Preliminary FCM studies of the effect of TAM on MCF-7 tumors in nude mice showed in accordance with the present results (Fig. 2B) that the proliferation kinetics rapidly reached a new steady state, indicated by stable levels of cell cycle fractions following treatment. Thus, the PLM investigations of the TAM-treated MCF-7 tumors were justified, and they were initiated 8 days after the first TAM treatment when the steady state was reached. The cell cycle fractions were well determined by FCM, and the S-phase fractions were in addition confirmed by the LI determinations (Table 1). Also the $T_{D50}$ were calculated from sufficient growth data.

The PLM data of all three groups of tumors fitted well to the first peak which provided a reliable determination of the $T_s$ and $T_G_1$ (Fig. 3). Beyond the first peak the data did show a second rise, indicating cycling of labeled cells. The computer fit, however, was very poor. Hence, the determination of $T_G_1$ and of $T_c$ was not very reliable. This is also indicated by the large SDs of the $T_G_1$ values (Table 1) and by the broad frequency distributions of $T_c$ (Fig. 3).

Consequently, the calculated $T_{pop}$ and $\Phi$ values were accurately determined and thus considered valid, since the calculations were based only on the reliable parameters ($T_s$, LI/S-phase fraction, $T_D$, and $\lambda$) which are dependent on $T_s$ and $T_G_1$ (24). In contrast, the calculated $a$ and $G_1$ values (Table 1) were derived from $T_c$ and $T_{pop}$ which were less accurately determined and are therefore less reliable.

Based on the evaluation of the validity of the obtained kinetic parameters, it is concluded that the present in vivo investigation of the cell kinetics showed that, although TAM treatment of MCF-7 tumors grown in nude mice resulted in significant...
tumor regression, the treatment had no systematic effect on the distribution of cells in the cell cycle phases, and it did not significantly change the traverse of the cells through the cell cycle. As a consequence, the results indicate that the treatment-induced tumor regression was caused by an increase in the cell loss. This interpretation of the data is strongly supported by the 1.0-mg estradiol PLM data which showed a Tc value very similar to the Tc value in TAM-treated tumors, but the cell loss factor was similar to that found for the 0.1-mg estradiol group and thus significantly lower than that in the TAM group. Cell loss increases with increasing tumor size, but the present PLM investigations were performed on tumors of approximately the same size. Thus, the increase in cell loss can be taken as an indication of a real increase after TAM treatment.

The present in vivo FCM data showed some variation in the cell cycle distribution of individual tumors (Fig. 2). This involves the risk that small treatment-induced changes are lost in the intertumor variation. However, no systematic changes were observed, and in particular the data showed no indication of any block with G1 accumulation in the cell cycle as that found under in vitro conditions. Another difficulty in the interpretation of the FCM data of treated tumors is the possible presence of “doomed” cells, i.e., treatment-inactivated cells that go through a few cell cycles before they die. The FCM and PLM provide no information on the amount of doomed cells. The MCF-7 in vivo data correspond to those previously reported on the T61 human breast cancer grown in nude mice (8). Only one other study has used the PLM assay for the evaluation of hormone effect on breast cancer cells (25). This study was related to the effect of hormone withdrawal on mouse mammary tumors. The PLM data were interpreted as showing that hormone deprivation was accompanied by a decrease in growth fraction and a decrease in cell production rate due to an increase in the mean cycle transit time, particular in the G1 phase. The rate of cell loss was found unchanged. Thus, the cell kinetic data indicate a difference in the mechanism of action between hormonal withdrawal and tamoxifen treatment. This is supported by the described oophorectomy sensitivity yet tamoxifen resistance of the Br-10 human breast cancer grown in nude mice (26), and the oophorectomy resistance yet tamoxifen sensitivity of the T61 heterotransplanted human breast cancer (26).

Cell loss is a biological feature which takes place in normal as well as in neoplastic tissues (20, 24). In most normal tissues an equilibrium exists between cell proliferation and cell loss. Another difficulty in the interpretation of the FCM data of treated tumors is the possible presence of “doomed” cells, i.e., treatment-inactivated cells that go through a few cell cycles before they die. The FCM and PLM provide no information on the amount of doomed cells. The MCF-7 in vivo data correspond to those previously reported on the T61 human breast cancer grown in nude mice (8). Only one other study has used the PLM assay for the evaluation of hormone effect on breast cancer cells (25). This study was related to the effect of hormone withdrawal on mouse mammary tumors. The PLM data were interpreted as showing that hormone deprivation was accompanied by a decrease in growth fraction and a decrease in cell production rate due to an increase in the mean cycle transit time, particular in the G1 phase. The rate of cell loss was found unchanged. Thus, the cell kinetic data indicate a difference in the mechanism of action between hormonal withdrawal and tamoxifen treatment. This is supported by the described oophorectomy sensitivity yet tamoxifen resistance of the Br-10 human breast cancer grown in nude mice (26), and the oophorectomy resistance yet tamoxifen sensitivity of the T61 heterotransplanted human breast cancer (26).

The present investigation does not yield information on the in vivo mechanisms of TAM. However, biological modification of cell loss instead of cell proliferation might represent a future strategy in cancer treatment. It has now been demonstrated in two individual heterotransplanted human breast carcinomas (8) that the in vivo growth-inhibitory effect of TAM does not include an accumulation of cells in the G1 phase of the cell cycle. Thus, clinical studies aiming at enhancing the cytotoxic effect of S-phase-specific cytotoxic drugs by pretreatment with TAM and estrogen (4–6) should be reconsidered.

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REFERENCES


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