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# Contrasting Ability of Antiestrogens to Inhibit MCF-7 Growth Stimulated by Estradiol or Epidermal Growth Factor

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**Abstract**—*A potential mechanism is described by which a growth factor may prevent the action of antiestrogens or reactivate the growth of hormone-responsive breast carcinoma in patients undergoing tamoxifen (TAM) treatment. Epidermal growth factor (EGF)-stimulated growth ($10^{-8}$ M EGF) was assayed in the MCF-7 breast cancer cell line in the presence of various concentrations ($10^{-10}$ to $10^{-6}$ M) of three antiestrogens, 4-hydroxytamoxifen (OH TAM), TAM and ICI 164384. In each case, the EGF-stimulated increases in DNA were not inhibited by the antiestrogen. OH TAM and ICI 164384 inhibited estradiol ($E_2$) stimulated cell proliferation in a dose-related fashion. However, in the presence of both $E_2$ and EGF, these two antiestrogens inhibited $E_2$ effects only; EGF promotion of growth was unaffected. Pretreatment of MCF-7 cells for 2 days with either OH TAM or ICI 164384 did not inhibit EGF-induced increases in cell proliferation. We propose that eventual antiestrogen therapeutic failure may be caused by the paracrine influences of growth factors from neighboring cells.*

## INTRODUCTION

APPROXIMATELY one-third of breast cancer patients have hormone-dependent tumors [1]. Tamoxifen (TAM), an antiestrogen with widespread clinical use, is an effective treatment in these women, but therapy is not effective in all estrogen receptor (ER)-positive patients. Unfortunately, during prolonged antiestrogen therapy, even the patient with responsive tumors can be expected eventually to become resistant [2].

Recently, breast cancer cell lines have been shown to have a receptor to epidermal growth factor (EGF) EGFR [3–5], and to proliferate in response to added EGF [3, 4, 6, 7]. Many breast cancer cell lines, both ER-positive and -negative, secrete EGF-like polypeptides [7–9] which may have both an autocrine and paracrine effect on cells [10–12]. Since hormone-responsive breast cancers contain a mixture of neighboring estrogen-dependent and independent cells [13], then resistance to antiestrogen therapy may occur if growth is stimulated in ER-positive cells by paracrine-acting growth factors from ER-negative cells [2].

In the MCF-7 breast cancer cell line, estradiol ($E_2$) selectively enhances the secretion of growth-promoting activity into culture media [14]. EGF-like and insulin-like growth factor I (IGF-I)-like polypeptides have been identified [7, 8, 15]. Acid-extracted conditioned media free of $E_2$ has also been found stimulatory for MCF-7 tumor formation in ovariectomized athymic mice [16]. Since antiestrogen binding to ER may prevent the stimulation of positive growth factor activity, we posed the question whether the addition of EGF to culture media was sufficient to cause an increased MCF-7 cell proliferation in the presence of antiestrogens [2, 17]. A subsequent concurring preliminary report by Koga and Sutherland using T47D cells found that antiestrogens did not inhibit EGF-stimulated growth [18]. However, the recent study by Vignon and coworkers [19] suggests that antiestrogens can inhibit growth factor-stimulated proliferation via a mechanism dependent upon ER. $E_2$ reverses the effect.

We have followed up our original reports [2, 27] to evaluate the effectiveness of a range of antiest-

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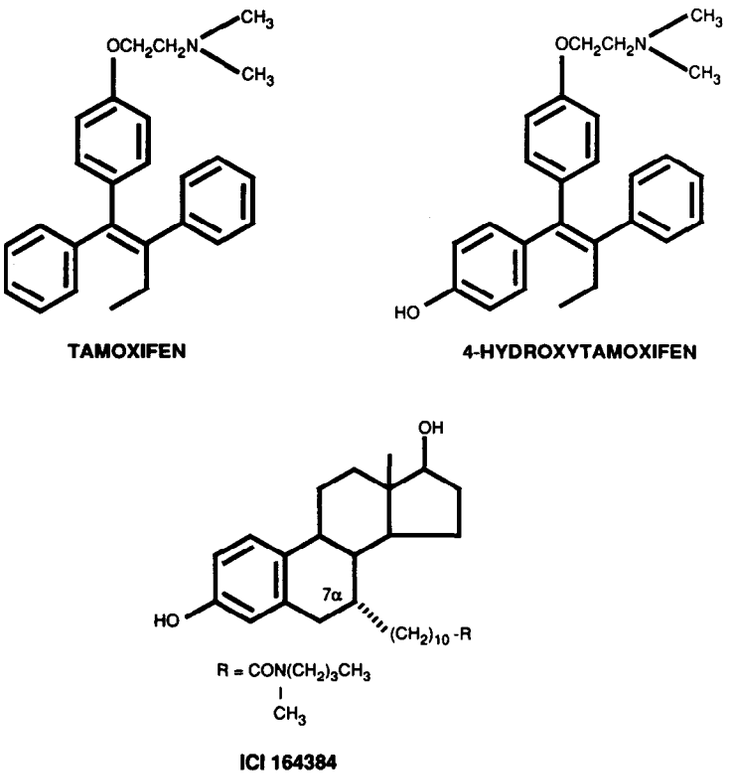


*Fig. 1. Chemical structures of tamoxifen, 4-hydroxytamoxifen and ICI 164384.*

rogen with different properties (Fig. 1). TAM was selected because the drug is used as the front-line endocrine therapy for breast cancer [20]; OH TAM was tested because it is a potent metabolite of TAM with high affinity for the ER [21]. However, both of these compounds have some agonist activities in rats and mice as defined by the 3-day uterotrophic assay. Also, in our hands and as reported by others, low concentrations of these compounds in tissue culture are growth stimulatory [23, 24]. Since the agonist activities of these antiestrogens may prime MCF-7 cells to the effects of EGF, we also tested a newly described antiestrogen, ICI 164384, which is totally devoid of estrogenic activity in both the rat and mouse uterus and also in MCF-7 breast cancer cells [25].

Phenol red, a pH indicator in tissue culture medium, has been found to be a weak estrogen agonist [26]. Our original observations, and those of Koga and Sutherland, were conducted in media containing phenol red [17, 18]. It is not clear what role estrogens might play in the sensitivity of MCF-7 cells to EGF. We, therefore, used a culture system free of phenol red and endogenous estrogens found in serum. Under phenol red-free conditions, we have now demonstrated that the antiestrogens tested were not able to inhibit proliferation caused by EGF and that EGF is capable of reversing antiestrogen-inhibited MCF-7 growth.

## MATERIALS AND METHODS

*Materials*

EGF was obtained from Collaborative Research (Bedford, MA). OH TAM, TAM and ICI 164384 were gifts from ICI plc (Pharmaceuticals Division, Macclesfield, U.K.). $E_2$ was obtained from Sigma (St. Louis, MO).

*Cell culture*

MCF-7 cells were obtained from Dr. Dean Edwards (San Antonio, TX) who originally obtained the stock from the Michigan Cancer Foundation. These cells were karyotyped as MCF-7 cells by Dr. Lorraine Meisner of the University of Wisconsin. MCF-7 cells were grown in minimum essential medium (MEM) with 0.29 mg L-glutamine/ml, 100 units penicillin/streptomycin/ml, 6 ng insulin/ml, 0.35 g $NaHCO_3$/l, 25 mM hepes and 5% charcoal-stripped calf serum. Media components were obtained from Gibco (Grand Island, NY). Cells were harvested by an initial wash with CMFH followed by a 2-min incubation with a 0.25% trypsin solution containing 1 mM EDTA.

*DNA growth assay*

MCF-7 cells were plated at a density of 14,000 cells/well in 24-well culture plates in phenol red-free media (Sigma). Media was changed each day

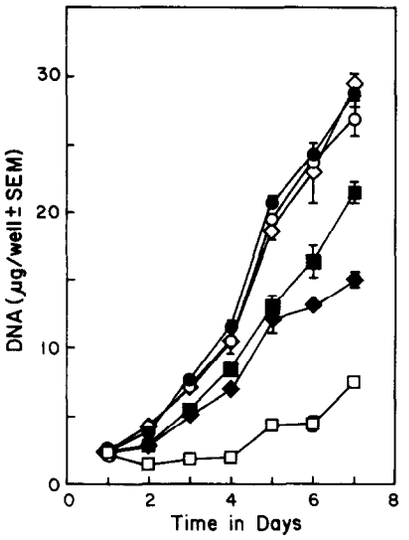


*Fig. 2. $E_2$-stimulated growth of MCF-7 cells. MCF-7 cells were treated with different concentrations of $E_2$ for 7 days. DNA values ± S.E.M. were determined for each of the 7 days. $E_2$ $10^{-13}$ M (◆), $E_2$ $10^{-12}$ M (■), $E_2$ $10^{-11}$ M (◇), $E_2$ $10^{-10}$ M (●), $E_2$ $10^{-9}$ M (○), control (□).*

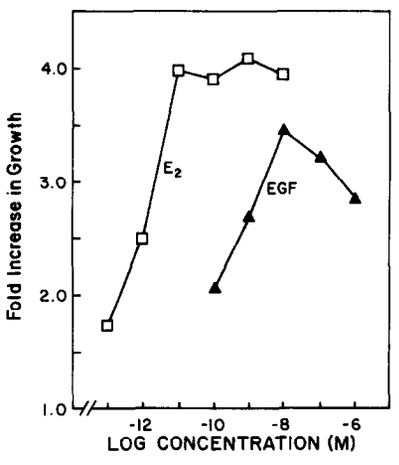


*Fig. 3. EGF vs. $E_2$ stimulation of MCF-7 cells. MCF-7 cells were grown for 7 days in the presence of various concentrations of EGF or $E_2$. DNA values were determined and plotted as fold increase in growth over control growth. Control DNA levels were 5.27 ± 0.95.*

for 2 days after plating and compounds were added 4 days after plating. Media containing compounds was changed every other day for the 6 days of the growth assay. DNA was measured fluorometrically using Hoechst 33258 (Calbiochem-Behring) according to La Barca and Paigen [27].

## RESULTS

*Growth sensitivity of MCF-7 cells*

In the absence of phenol red, $E_2$ is a very potent growth stimulator of MCF-7 cells (Fig. 2). After 7 days of treatment, the $ED_{50}$ was approx. $5 \times 10^{-13}$ M $E_2$. Maximum increases in DNA were obtained at an $E_2$ concentration of $10^{-10}$ M, which was the concentration used for subsequent experiments. EGF also promoted cell growth in this culture system, although it was not as stimulatory as $E_2$ (Fig. 3). A maximum 3.5-fold growth increase was obtained in an EGF concentration of $10^{-8}$ M. EGF-induced cell proliferation in our experiments usually ranged between 2.0-fold to 3.5-fold over controls. Maximum stimulation of growth was obtained after 6 to 8 days of EGF treatment (data not shown).

*Antiestrogen effect on EGF-stimulated growth*

EGF ($10^{-8}$ M) was added with various concentrations of TAM or OH TAM to determine whether EGF could overcome the growth inhibitory effects of these two antiestrogens (Fig. 4A and 4B). If added alone, both antiestrogens at high concentrations retained the *status quo* control growth. Interestingly, at concentrations corresponding to the approximate $K_d$ of these compounds for the ER, $10^{-8}$ M for TAM and $10^{-10}$ M for OH TAM, an increase in growth could be observed. This growth induction exemplifies the intrinsic agonist activities of these two compounds. However, below $10^{-8}$ M, TAM appears to be inactive. When EGF is also added, MCF-7 cell proliferation is induced. The increase in DNA is the same as that stimulated by EGF when added alone, with the exception of antiestrogen concentrations which displayed estrogenic activity. In the latter case, growth stimulation was additive.

To circumvent the problem of interpreting experimental data under conditions in which the usage of partial agonists has created a phenol red-like environment, a pure antiestrogen, ICI 164384, was also tested (Fig. 4C). Although we observed no agonist activity of this compound when tested alone, it was totally ineffective against inhibiting EGF-stimulated growth. Therefore, none of the antiestrogens used, regardless of the amount of intrinsic estrogenicity, was able to prevent increases in DNA caused by EGF treatment.

*$E_2$ and EGF effects on antiestrogen-inhibited growth*

We tested the antiestrogenicity of ICI 164384 and OH TAM. Both compounds decreased $E_2$ growth stimulation in a concentration-related fashion (Fig. 5A). But if EGF is also added, neither antiestrogen decreases growth below the level of DNA increases stimulated by EGF alone (Fig. 5B). These results argue against a common biochemical pathway for activated ER and EGFR. However, since compounds are added simultaneously, we could not discount the possibility that EGF could turn on EGFR before the antiestrogen became effective. Therefore, MCF-7 cells were pretreated for 2 days with OH TAM, an antiestrogen with high affinity for ER or ICI 164384, a pure antiestrogen, in an attempt to desensitize the cells to subsequent EGF treatment. Figure 6 illustrates that pretreatment with antiestrogens does not make MCF-7 cells refractory to EGF growth promotion.

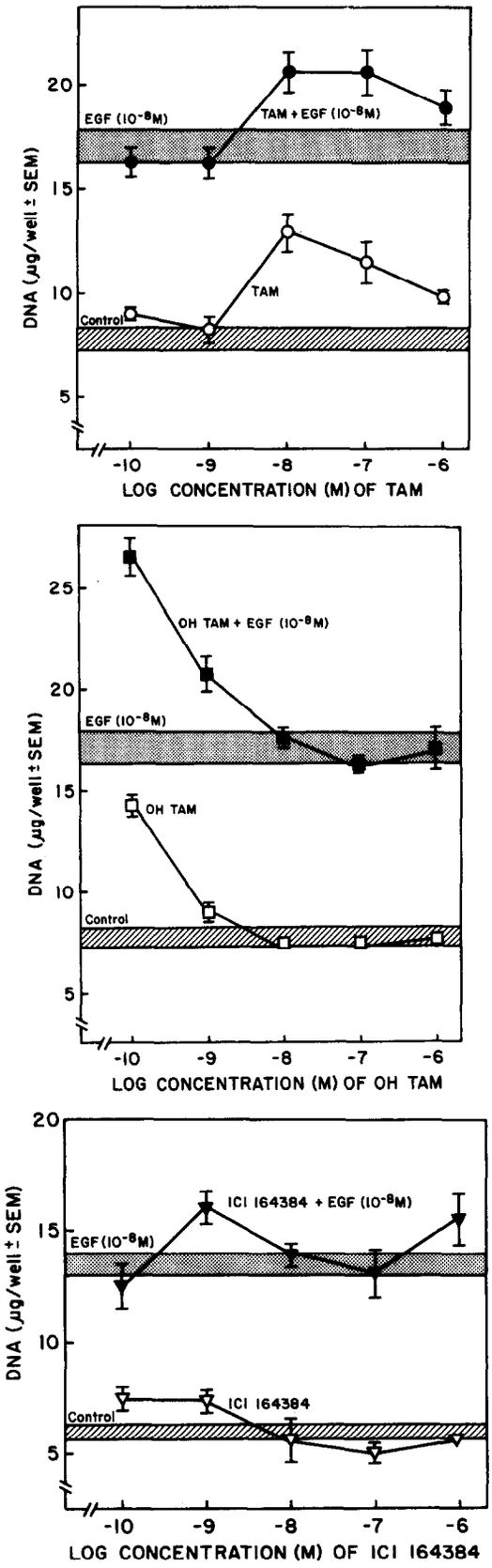


*Fig. 4. EGF stimulation of growth in the presence of TAM, OH TAM, or ICI* 164384. *MCF-7 cells were grown for 7 days in the presence of various concentrations of the antiestrogens with or without EGF* ($10^{-8}$ M): (*A*) *TAM* (*B*) *OH TAM or* (*C*) *ICI* 164384. *DNA determinations ± S.E.M. were made for each concentration including control (hatched bar) and EGF (stippled bar).*

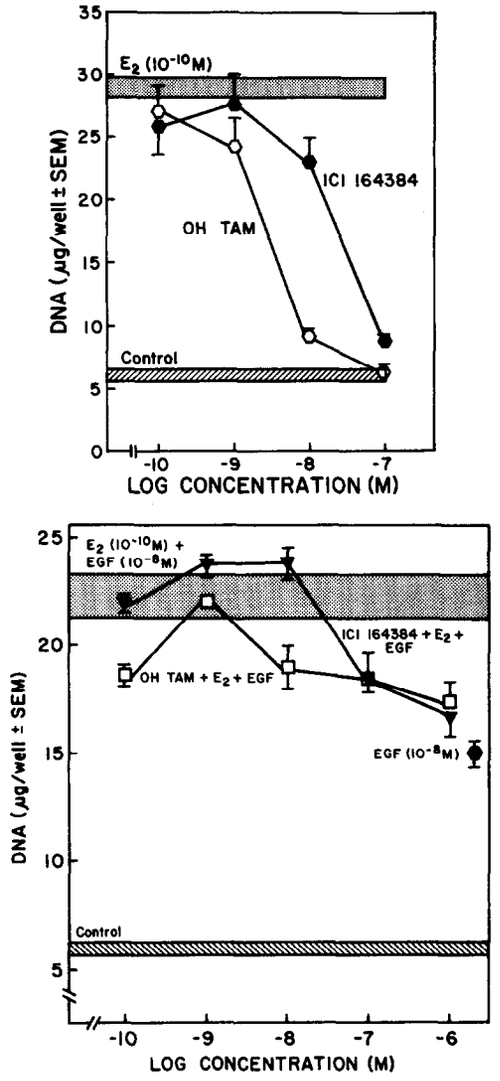


*Fig. 5. EGF stimulation of growth in the presence of OH TAM or ICI* 164384. (*A*) *MCF-7 cells were grown for 7 days in the presence of various concentrations of the antiestrogens OH TAM* ( ○ ), *or ICI* 164384 ( ● ) *plus* $E_2$ ($10^{-10}$ M). (*B*) *EGF* ($10^{-8}$ M) *and* $E_2$ ($10^{-10}$ M) *were added to increasing concentrations of OH TAM* (□) *or ICI* 164384 (▼). *DNA determinations ± S.E.M. were made for each concentration including control,* $E_2$, *EGF, and EGF +* $E_2$ *treatment.*

## DISCUSSION

MCF-7 breast cancer cells secrete many growth-promoting activities into media [28]. While $E_2$ treatment of MCF-7 cells enhances secretion of growth stimulators, antiestrogen treatment, on the other hand, stops $E_2$ effects by blocking $E_2$ binding to its receptor and possibly by, as has been recently reported, increasing the concentration of TGF-β [29]. TGF-β inhibits the growth of many different cell lines including MCF-7 [12, 29]. Therefore, antiestrogens may both decrease the level of positive growth factor and increase that of a growth inhibitory factor.

Recent experiments by Vignon *et al.* on MCF-7 cells grown without phenol red have shown an ER-mediated inhibition of EGF-stimulated growth in

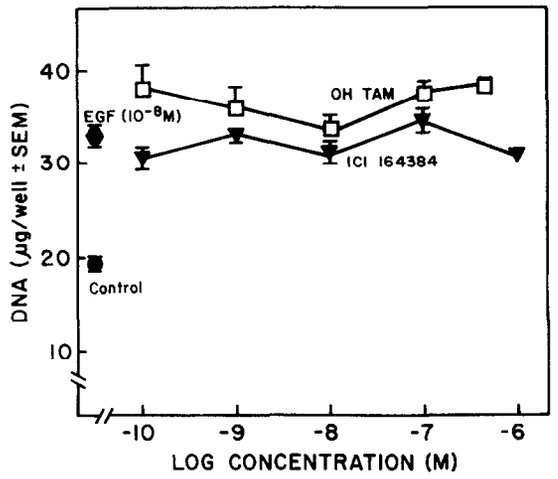


*Fig. 6. Antiestrogen pretreatment. MCF-7 cells were grown for 2 days in the presence of different concentrations of OH TAM (□) or ICI 164384 (▼). After 2 days, MCF-7 cells were treated additionally with EGF ($10^{-8}$ M) for 6 days. DNA determinations ± S.E.M. were made for each group including control (●) and EGF ($10^{-8}$ M) alone ( ⬢ ).*

*Table 1. Effect of OH TAM or ICI 164384 on the growth of MCF-7 cells*

| | µg DNA* | |
|---|---|---|
| | $n = 6$ | |
| Log molar concentration | OH TAM | ICI 164384 |
| Control 7.03 ± 0.38 | | |
| −10 M | 12.23 ± 1.0† | 7.90 ± 0.24 |
| − 9 M | 8.30 ± 0.45 | 7.93 ± 0.32 |
| − 8 M | 6.80 ± 0.33 | 6.70 ± 0.52 |
| − 7 M | 6.90 ± 0.30 | 5.90 ± 0.49 |
| − 6 M | 7.10 ± 0.37 | 6.53 ± 0.43 |

*Mean ± S.E.M.
†$P < 0.001$.
Statistical comparison of treated groups versus control was done by the Mann–Whitney test.

the presence of the antiestrogen OH TAM [19]. In contrast, our original report [2, 17] and that of Koga and Sutherland [18] using breast cancer cells cultured in media containing phenol red, did not find that antiestrogen inhibit growth factor-stimulated increases in DNA. Since it is not clear what effect phenol red has on EGF promotion of growth, we repeated our experiments in culture media without phenol red.

We tested three antiestrogens under phenol red-free conditions. The triphenylethelene, TAM, and its hydroxylated derivative, OH-TAM, were used because of their clinical significance [20]. Both of these compounds have some intrinsic estrogenic activity and for this reason, a steroidal, pure antiestrogen, ICI 164384, was also tested. These three antiestrogens represent a variety of binding affinities for the ER. OH TAM has the highest relative binding affinity with a RBA = 252 ($E_2$ = 100) in the rat uterine cytosol competitive assay [30]. TAM has a RBA = 3 and ICI 164384 has reported an 8 times greater binding affinity than TAM in this assay [25, 30]. However, none of the three antiestrogens offered any advantage in decreasing EGF-promoted increases in DNA despite differences in intrinsic estrogen activity, structure or relative binding affinity for the ER.

It is not likely that EGF actions in our assay system share a common growth activation pathway with ER. First, the antiestrogens tested do not inhibit EGF stimulation. OH TAM and ICI 164384 are antiestrogenic in the presence of $E_2$ but do not decrease growth if EGF is also present. Finally, if EGF-induced growth can be mediated through the ER growth activation pathway, then a 2-day pretreatment with antiestrogen before the addition of EGF should inactivate ER. We have also shown that cells do not become refractory to the effects of EGF if pretreated with either OH TAM or ICI 164384.

When used singly, EGF, in comparison to $E_2$ treatment, does not stimulate cell growth maximally. However, EGF-stimulated growth might be maximized by the addition of other factors. Recent reports have shown a possible cyclic mechanism of supporting growth interactions between epithelial breast cancer cells and the surrounding stromal elements. Human breast cancer cells in long-term tissue culture secrete factors related to IGF-1, EGF and platelet-derived growth factor (PDGF) into culture medium [29, 31, 32]. EGF and IFG-I are growth stimulatory to both epithelial and fibroblast cells [12, 33, 34]. Although PDGF does not increase DNA synthesis of epithelial cells since these cells do not express PDGF receptors, PDGF is chemotactic for fibroblast and is a very potent mitogen for these cells [35, 36]. Interestingly, PDGF binding to fibroblasts stimulates the secretion of IGF-I, a very potent mitogen for breast cancer cells [37, 38].

EGF has been shown to act at a discrete restriction point in $G_1$ while other growth factors such as PDGF and IGF-1 act at an earlier and later point, respectively, in $G_1$ to promote cell growth [39]. Tamoxifen inhibits the MCF-7 cell cycle at $G_1$ [40, 41]. If antiestrogens inhibit estrogen-stimulated growth factor production, the addition of EGF might be expected to cause a progression through the restriction point. By contrast, the constant presence of EGF throughout the cell cycle and the requirement for additional growth factors may complicate the cell's interpretation of the growth signal. This would explain the lower DNA levels when EGF is used alone or combined with $E_2$.

Continuous antiestrogen therapy has been shown

to be an optimum treatment for breast cancer [42, 43]; however, this cannot be considered to be a cure since breast cancer therapy eventually does fail. In this paper, we have demonstrated that antiestrogens do not control replication produced by EGF in a cell system *in vitro*. These results illustrate the possibility that exogenous growth factors in a tumor milieu may continue to stimulate replication by breast tumor cells during antiestrogen therapy.

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