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Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant*

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Abstract

We seek to evaluate the clinical consequences of resistance to antihormonal therapy by studying analogous animal xenograft models. Two approaches were taken. 1) MCF-7 tumors were serially transplanted into selective estrogen receptor modulator (SERM) -treated immunocompromised mice to mimic five years of SERM treatment. The studies *in vivo* were designed to replicate the development of acquired resistance to SERMs over years of clinical exposure. 2) MCF-7 cells were cultured long-term under SERM-treated or estrogen withdrawn conditions (to mimic aromatase inhibitors), and then injected into mice to generate endocrine-resistant xenografts. These tumor models have allowed us to define Phase I and Phase II antihormonal resistance according to their responses to E₂ and fulvestrant. Phase I SERM-resistant tumors were growth stimulated in response to estradiol (E₂), but paradoxically, Phase II SERM and estrogen withdrawn-resistant tumors were growth inhibited by E₂. Fulvestrant did not support growth of Phase I and II SERM-resistant tumors, but did allow growth of Phase II estrogen withdrawn-resistant tumors. Importantly, fulvestrant plus E₂ in Phase II antihormone-resistant tumors reversed the E₂-induced inhibition and instead resulted in growth stimulation. These data have important clinical implications. Based on these and prior laboratory findings, we propose a clinical strategy for optimal third-line therapy: patients who have responded to and then failed at least two antihormonal treatments may respond favorably to short-term low-dose estrogen due to E₂-induced apoptosis, followed by treatment with fulvestrant plus an aromatase inhibitor to maintain low tumor burden and avoid a negative interaction between physiologic E₂ and fulvestrant.

Keywords

Breast Cancer; Estradiol; Tamoxifen; Raloxifene; Fulvestrant

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1. Introduction

The target for endocrine therapy is the estrogen receptor (ER), and the translation of laboratory findings on the control of estrogen-regulated tumor growth has established the current treatment strategies which have been validated in clinical trials [1–7]. Tamoxifen (TAM), the prototype selective estrogen receptor modulator (SERM), is a current standard adjuvant treatment used for 5 years in all stages of ER-positive breast cancer [8–12]. However, aromatase inhibitors (AIs) are becoming the leading choice for antihormonal treatment of ER-positive breast cancer in postmenopausal patients. Still, there is a need to study the long-term therapeutic consequences of TAM because of its use in premenopausal ER-positive breast cancer [9,10], and as a chemopreventive agent to reduce the risk of breast cancer in high-risk women [13]. There is also considerable interest in the use of raloxifene (RAL), a related SERM, as a chemopreventive agent [14,15], since it has recently been shown in the STAR trial (Study of TAM and RAL) to exhibit equivalent efficacy as TAM in reducing the risk of breast cancer [16]. Additionally, RAL is used for the treatment and prevention of osteoporosis in postmenopausal women [17], has been noted to have endometrial safety [14,15,17], and reduces the risk of cardiovascular disease [18–20]. Since RAL may have to be given indefinitely to prevent osteoporosis, RAL-exposed breast cancer will almost certainly occur. Overall, there is a large and growing population of women at risk for developing endocrine therapy-resistant breast cancer.

It is important to emphasize that the successful treatment of patients with one endocrine agent and then failure, leads to exhaustive endocrine therapy with the succession of agents, each with decreasing efficacy. The failure of TAM as a first-line therapy forms the basis of the use of AIs or fulvestrant (ICI 182,780, Faslodex[®]) as second-line therapies for the treatment of breast cancer.

In postmenopausal women, the aromatase enzyme converts androgens to estrogens in peripheral tissues such as adipose tissue and in the breast cancer tissue itself [21,22]. AIs block activity of this enzyme and fall into two classes, steroidal and non-steroidal [23–25]. Exemestane (Aromasin[®]) [26,27], a steroidal AI, irreversibly binds aromatase at the catalytic site and inactivates the enzyme. Anastrozole (Arimidex[®]) [28,29] and letrozole (Femara[®]) [30,31], non-steroidal AIs, bind aromatase at a different site, a heme group, to reversibly inhibit the enzyme. AIs have been evaluated in advanced breast cancer and in the adjuvant setting [26–31]. In the largest adjuvant trial, the ATAC trial (Arimidex, TAM, Alone or in Combination), patients in the anastrozole arm versus the TAM arm showed significantly longer disease-free survival, reduced contralateral breast cancer, and reduced distant metastases [32, 33]. Indeed, AIs are now recommended and may replace TAM as the standard first-line antihormonal adjuvant therapy in postmenopausal ER-positive breast cancer patients. Further, due to the success of this and of other trials evaluating AIs for extended adjuvant therapy, AIs are also indicated after 5 years [34] and even 2 years of TAM [26,29].

FUL is an analogue of E₂ and the first in a new class of drugs that are complete antiestrogens, that is, they display no agonist activity via AF-1 or AF-2 of the ER [35,36]. FUL also leads to potent downregulation of ER protein expression because FUL binding to ER induces an abnormal conformation that results in accelerated ubiquitylation and shuttling of the ER to the proteasome for degradation [35,37]. Two large Phase III clinical trials have been conducted to evaluate FUL versus the AI anastrozole in postmenopausal advanced ER-positive breast cancer patients who have failed TAM. Both of these trials showed that FUL was equally effective as anastrozole in terms of time to progression and objective response rates [38,39]. Hence, FUL has been approved as a second-line therapy. FUL is also currently being evaluated in combination with AIs [36].

Over the past two decades, we have developed unique MCF-7 breast cancer xenograft models of long-term SERM (TAM and RAL) treatment and models of long-term estrogen withdrawal that could reasonably mimic resistance to AIs. These tumor models were developed *in vivo* and *in vitro*. The *in vivo* tumor models were designed to mimic the selection process needed over years to develop acquired resistance in the clinic by serially implanting MCF-7 tumors into SERM-treated and ovariectomized immunodeficient mice also over a period of years [40–50]. The *in vitro* tumor models were developed by culturing MCF-7 cells in estrogen-free conditions, with or without SERM treatment if appropriate, for over 1 year to develop antihormone resistance, and then injecting these cells into ovariectomized athymic mice treated with the SERM, if appropriate, and allowing tumors to grow [51–53]. We now have in hand a panel of breast cancer xenograft and tissue culture models that have allowed us to define the evolution of resistance to antihormonal therapy into at least two phases, each of which exhibits distinct growth responses to E₂ and FUL. We found that the growth of Phase I SERM-resistant tumors is stimulated by E₂, while growth of Phase II SERM or estrogen withdrawn-resistant tumors is, paradoxically, inhibited by E₂ treatment. Previous studies conducted by our group have shown that E₂ not only inhibits growth of Phase II SERM and estrogen withdrawn-resistant tumors; it also induces apoptosis, leading to tumor regression. However, a fraction of these Phase II tumors eventually re-grow after E₂-induced regression occurs, but these tumors are again re-sensitized to antihormonal therapy. We also found that while FUL does not support the growth of Phase I and II SERM-resistant tumors, it does allow growth of Phase II estrogen withdrawn-resistant tumors. Further, we found that while E₂ blocked growth of Phase II antihormone-resistant tumors, the combination of E₂ plus FUL resulted in robust growth. Phase II antihormonal resistance has not yet been widely recognized, but could be exploited by developing a novel third-line treatment plan based on short-term low-dose estrogen to debulk patients' tumors who fail exhaustive endocrine therapy, followed by the combination of FUL plus an AI to maintain low tumor burden and avoid a negative interaction between physiologic E₂ and FUL.

2. Materials and methods

2.1. Athymic mice, tumor inoculation, and tumor tracking

All procedures involving animals have been approved by the Fox Chase Cancer Center's Internal Animal Care and Use Committee.

All animal studies employed female ovariectomized athymic BALB/c nude (*nu/nu*) mice (Taconic, Hudson, NY, USA) that were inoculated with tumor cells at 5–6 weeks of age. For experiments employing tumor models which were generated and serially propagated as xenografts (*in vivo*), 1 mm³ tumor sections were bilaterally transplanted using a trochar into the axillary mammary fat pads. For studies using tumor models which were generated and maintained in tissue culture (*in vitro*), cells were suspended in phosphate-buffered saline and bilaterally injected into axillary mammary fat pads at 10⁷ cells per site.

Tumor growth was tracked by weekly measurements of tumor length (*l*) and width (*w*) using Vernier calipers, from which the tumor cross-sectional area was calculated using the equation: $(l/2) \times (w/2) \times \pi$. Tumor growth curves are expressed as the average cross-sectional tumor area per treatment group $\pm$ standard error (SE).

2.2. Drug treatments

Mice were treated with estrogen by implanting a 0.3 cm E₂ silastic capsule subcutaneously into the intrascapular region on the back of the mouse at the time of tumor cell inoculation. The capsules were prepared by filling silicone tubing (0.078 inch inner diameter/ 0.125 inch outer diameter; Fisher) 0.3 cm in length with a 1:3 (*w/w*) mixture of E₂ (Sigma-Aldrich, St.

Louis, MO, USA) and silastic elastomer (Dow Corning, Midland, MI, USA), and then sealing the ends with silicone adhesive (Dow Corning) and sterilized by gamma irradiation. Athymic mice implanted with these capsules achieve mean serum levels of 83.8 pg/ml (308 pM) E₂ [54], which approximates perimenopausal E₂ levels in women. RAL and TAM were orally administered by gastric intubation at 1.5 mg/day 5 days per week. Evista tablets (Eli Lilly Pharmaceuticals, Indianapolis, IN, USA; purchased from the Fox Chase Cancer Center's pharmacy), the clinically available form of RAL (60 mg/tablet), were initially dissolved in water, and then suspended at 10 mg/ml in 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol 400, 0.5% Tween 80) and 0.9% carboxymethyl cellulose. TAM (Sigma) was initially dissolved in ethanol (EtOH), and then suspended at 10 mg/ml in 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol 400, 0.5% Tween 80) and 0.9% carboxymethyl cellulose. FUL was administered by *sc* injection in the scruff of the neck at a total of 10 mg/week. For the experiment depicted in Figure 1, four different FUL formulations and dosing schedules were used: FUL was initially dissolved in 1) EtOH or 2) dimethylsulfoxide (DMSO), and then made into a suspension with peanut oil at 50 mg/ml and administered as a 5 mg injection twice per week; 3) FUL was dissolved in only DMSO at 50 mg/ml and administered as a 2 mg injection 5 days per week; or 4) the clinical faslodex preparation, a 50 mg/ml proprietary solution of FUL in primarily EtOH supplemented with castor oil as a release rate modifier, was administered as 2 mg injections 5 days per week. For all other experiments, only the clinical Faslodex preparation was used and administered as 2 mg injections 5 days per week. FUL powder was a kind gift of AstraZeneca (Macclesfield, United Kingdom), and the clinical Faslodex preparation was purchased from the Fox Chase Cancer Center's pharmacy.

2.3. Generation of MCF-7/E2 xenograft tumors

The MCF-7/E2 xenograft tumor model, representing the antihormonal-sensitive stage of breast cancer, was originally developed by bilateral injection of 10⁷ MCF-7 cells, grown in tissue culture, into the axillary mammary fat pads of female ovariectomized athymic BALB/c *nu/nu* mice implanted with a 0.3 cm E₂ capsule [40]. The resulting MCF-7/E2 tumors have been propagated *in vivo* by serial transplantation into likewise E₂-treated ovariectomized athymic mice.

2.4. Generation of MCF-7/RAL1 xenograft tumors

The MCF-7/RAL1 (Phase I) SERM-resistant tumor model was derived by transplantation of MCF-7/E2 tumors into RAL-treated ovariectomized athymic mice. After extended RAL treatment, a small percentage of these tumors showed minimal but significant growth, and following re-transplantation into new RAL-treated ovariectomized athymic mice, these tumors then exhibited robust RAL-stimulated growth [50]. MCF-7/RAL1 tumors have been propagated *in vivo* for over 3 years by serial transplantation into RAL-treated ovariectomized athymic mice.

2.5. Generation of MCF-7/RAL2 xenograft tumors

The MCF-7/RAL2 (Phase II) SERM-resistant tumor model was developed *in vitro* by tissue culture of MCF-7 cells in estrogen-free medium supplemented with 1 μ M RAL for over 1 year [51]. For every experiment involving MCF-7/RAL2 tumors, cells were grown in culture, and then bilaterally injected into the axillary mammary fat pads of ovariectomized athymic mice at 10⁷ cells per site. MCF-7/RAL2 cells were maintained in culture in estrogen-free MEM plus 5% dextran-coated charcoal-treated calf serum (DCC-CS), 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 $\times$ non-essential amino acids (all media components from Invitrogen, Carlsbad, CA, USA).

2.6. Generation of MCF-7/TAM2 xenograft tumors

The MCF-7/TAM2 (Phase II) SERM-resistant tumor model was developed in a similar manner as the MCF-7/RAL1 tumor model, by initial transplantation of MCF-7/E2 tumor pieces into TAM-treated ovariectomized athymic mice, and repeated transplantation of minimally growing tumors into new TAM-treated mice until robust TAM-stimulated growth occurred. These MCF-7/TAM tumors passed through different phases of SERM resistance; they were initially stimulated to grow by both TAM and E₂ [41,55], but have evolved over 5 years of serial propagation *in vivo* to a stage in which only TAM, but not E₂, stimulates growth [47–49].

2.7 Generation of MCF-7 long-term estrogen-deprived cell culture models

MCF-7:ED cells were derived by maintaining a population of MCF-7 cells under estrogen-deprived conditions for >1 year to mimic AI treatment, and represent Phase I resistance to long-term estrogen withdrawal. MCF-7:ED cells were originally selected in phenol red-free MEM plus 5% dextran-coated charcoal-treated calf serum, but have more recently been maintained (this report) in phenol red-free RPMI, 10% dextran-coated charcoal-treated fetal bovine serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1× non-essential amino acids.

MCF-7/5C cells were clonally isolated from a population of MCF-7 cells grown under long-term estrogen-free conditions [56] and represent Phase II LTED resistance. MCF-7/5C cells were originally cultured in estrogen-free MEM plus 5% dextran-coated charcoal-treated calf serum, and under these conditions, MCF-7/5C cells exhibited estrogen and SERM independent growth [52,56]. However, we have observed that when MCF-7/5C cells are switched to estrogen-free RPMI plus 10% dextran-coated charcoal-treated calf serum, the cells undergo rapid apoptosis when treated with 1 nM E₂ [52,53]. In all experiments described in this report, MCF-7/5C cells were maintained under the latter media conditions (estrogen-free RPMI plus 10% dextran-coated charcoal-treated calf serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1× non-essential amino acids).

2.8 Generation of MCF-7/5C estrogen-deprived xenograft tumors

The MCF-7/5C (Phase II) long-term estrogen withdrawn-resistant tumors were generated by bilateral injection of these cells grown in culture into the axillary mammary fat pads of ovariectomized athymic mice at 10⁷ cells per site.

2.9 Cell proliferation assays

Wild-type MCF-7 cells were switched from phenol red-containing RPMI medium supplemented with 10% FBS to phenol red-free RPMI medium supplemented with 10% dextran-coated charcoal-treated fetal bovine serum for 4 days prior to beginning the proliferation assay. Since MCF-7/ED and MCF-7/5C cells are routinely maintained in estrogen-free media, no media switch was required. MCF-7, MCF-7/ED, and MCF-7/5C cells were seeded in estrogen-free RPMI containing 10% DCC-FBS at a density of 2×10⁴ cells per well in 24-well plates. After 24 hours (Day 0), the medium was replaced with fresh estrogen-free RPMI medium and cells were treated with 0.1% ethanol vehicle (control), 1 nM E₂, 10 nM FUL, or 1 nM E₂ + 10 nM FUL. Cells were retreated with the drugs on days 2, 4, and 6 and the experiment was stopped on day 7. The DNA content of the cells, a measure of proliferation, was determined using a Fluorescent DNA Quantitation kit (Bio-Rad Laboratories, Hercules, CA). For each analysis, six replicate wells were used, and at least three independent experiments were performed. Proliferation of cell lines following 7 day of growth are shown as the mean DNA amount per well per treatment ± standard deviation (SD).

2.10 Statistical analyses

In tumor growth experiments in which treatments were started at the time of tumor inoculation, tumors were analyzed longitudinally with two-factor analysis of variance (ANOVA) to determine significant differences in cross-sectional areas between all tumors in each treatment group in a time-dependent manner (data in Figures 1 through 4, and 6 through 7). In the tumor growth experiments in which treatments were started after the tumors were established, one-factor ANOVA was used to determine significant differences in cross-sectional areas between all tumors in each treatment group on the last day of the experiment (data in Fig. 8, day 52). All statistical tests were two-sided and calculated using SAS (SAS Institute, Cary, NC, USA).

3. Results

3.1 Growth of MCF-7/E2 tumors and responsiveness to FUL

MCF-7/E2 xenograft tumors are propagated *in vivo* by serial transplantation into 0.3 cm E₂ capsule-implanted ovariectomized athymic mice. To explore the sensitivity of MCF-7/E2 tumors to FUL, MCF-7/E2 tumor cores were implanted into 30 ovariectomized athymic mice and separated into 6 groups of 5 mice each, or 10 tumors per group. The treatment groups were control (no treatment), 0.3 cm E₂ capsule *sc*, or 0.3 cm E₂ capsule *sc* plus 1 of 4 different formulations and dosing schedules of FUL totaling 10 mg/week *sc* (Fig. 1). Two of the FUL formulations were suspensions made with peanut oil, differing by whether FUL was initially dissolved in EtOH or in DMSO. These FUL (EtOH/peanut oil or DMSO/peanut oil) suspensions were administered as 5 mg *sc* injections given two days per week, totaling 10 mg/week. The third formulation was FUL dissolved in only DMSO, and was administered as a 2 mg *sc* injection given five days per week, totaling 10 mg/week. The fourth FUL formulation corresponded to the clinical Faslodex preparation, which is a proprietary solution of primarily EtOH and some castor oil as a slow release-rate modifier. The clinical Faslodex preparation was administered as a 2 mg *sc* injection given 5 times per week, totaling 10 mg/week.

The MCF-7/E2 tumors grew robustly when treated with the 0.3 cm E₂ capsule, but did not grow in the control group (Fig. 1, E₂ vs. Control, $P < 0.0001$), demonstrating that these tumors were dependent on E₂. The implanted capsules produce E₂ levels that are in the physiologic range observed in perimenopausal women. The cross-sectional areas of each of the four groups of MCF-7/E2 tumors treated with E₂ plus FUL was significantly smaller than those tumors treated with E₂ alone (Fig. 1, all P -values < 0.0001). Therefore, FUL inhibited E₂-stimulated growth of MCF-7/E2 tumors. However, the degree of growth inhibition varied depending upon the formulation. Comparing the FUL suspensions in peanut oil given 2 times per week, FUL initially dissolved in DMSO inhibited tumor growth significantly better than FUL initially dissolved in EtOH (Fig. 1, $P = 0.001$). Comparing the FUL formulations given 5 times per week, FUL dissolved in only DMSO inhibited tumor growth significantly better than the clinical Faslodex preparation (Fig. 1, $P = 0.004$). Although we did not measure circulating FUL levels, we hypothesize that circulating levels of FUL were higher when using DMSO-based formulations, leading to more potent inhibition of E₂-stimulated tumor growth.

3.2 Growth of MCF-7/RAL1 tumors

MCF-7/RAL1 tumors are maintained *in vivo* by serial transplantation into 1.5 mg/day RAL-treated ovariectomized athymic mice. To illustrate the phase of SERM resistance the MCF-7/RAL1 tumor should be categorized into, MCF-7/RAL1 tumor cores were implanted into 20 ovariectomized athymic mice and separated into 4 treatment groups of 5 mice each (10 tumors/group) corresponding 1.5 mg/day RAL *po*, 0.3 cm E₂ capsule *sc*, 2 mg/day FUL *sc* (Faslodex preparation), and control (no treatment). The MCF-7/RAL1 tumors were significantly stimulated to grow by RAL treatment ($P < 0.0001$) and by E₂ treatment ($P < 0.0001$) compared

to control treatment (Fig. 2). However, a modest amount of growth was observed in the control-treated group, indicating that these tumors are not absolutely dependent upon an ER ligand with partial agonist activity. We have previously shown that primary cultures of MCF-7/RAL1 tumors exhibit equivalent levels of estrogen response element (ERE)-regulated reporter gene activity in the absence of E₂ as did primary cultures of MCF-7/E2 tumors when treated with E₂ [50]. Thus, the unliganded ER activity in MCF-7/RAL1 tumors is high and probably contributed to the modest growth of these tumors without the need of RAL or E₂. FUL did not significantly effect the growth of MCF-7/RAL1 tumors (Fig. 2). Thus, either a SERM or E₂, but not FUL, supports the growth of these MCF-7/RAL1 xenografts. Therefore, these tumors are categorized as Phase I SERM-resistant.

3.3 Growth of MCF-7/RAL2 tumors (Fig. 3)

MCF-7/RAL2 tumor cells are maintained *in vitro* by culture in media containing 1 μ M RAL. To study the growth properties of MCF-7/RAL2 cells *in vivo*, the cells were grown in culture and injected into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 (10 tumors/group) and treated with 1.5 mg/day RAL *po*, 0.3 cm E₂ capsule *sc*, 2 mg/day FUL *sc* (Faslodex preparation), or control (not treated). The MCF-7/RAL2 tumors only grew when treated with RAL (RAL vs. control, $P < 0.0001$), and did not form any palpable tumors by day 42 when treated with E₂, FUL or not treated (control) (Fig. 3). We have previously shown that when MCF-7/RAL2 tumors are allowed to grow by treating with TAM until they are established and then switching treatments to E₂, E₂ causes tumor regression by inducing apoptosis as measured by TUNEL staining [51]. Therefore, growth of the MCF-7/RAL2 tumors was dependent on RAL, but inhibited by E₂ and FUL, which categorizes these tumors as Phase II SERM-resistant.

3.4 Growth of MCF-7/TAM2 tumors

MCF-7/TAM2 tumors are propagated *in vivo* by serial transplantation into 1.5 mg/day TAM-treated ovariectomized athymic mice. To characterize the growth properties of this tumor type, MCF-7/TAM2 tumor cores were implanted into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 (10 tumors/group) and treated with 1.5 mg/day TAM *po*, 0.3 cm E₂ capsule *sc*, 2 mg/day FUL (Faslodex) *sc*, or not treated (control). MCF-7/TAM2 tumors were stimulated to grow by TAM compared to the control group (Fig. 4, $P < 0.0001$). The control group did show a minimal amount of growth (Fig. 4), which is hypothesized to be due to substantial unliganded ER activity as in the MCF-7/RAL1 model. FUL did not significantly effect growth of the MCF-7/TAM2 tumors versus control treatment. Interestingly, E₂ did significantly inhibit tumor growth compared to the control group (Fig. 4, $P = 0.0004$). As with the MCF-7/RAL2 tumors, we have previously demonstrated that E₂ treatment leads to regression of MCF-7/TAM2 tumors [47,49] by inducing apoptosis as detected by TUNEL staining [48]. Therefore, TAM stimulated growth, FUL did not support growth, and E₂ inhibited growth of MCF-7/TAM2 tumors, defining this model as Phase II SERM-resistant.

3.5 Growth of long-term estrogen withdrawn-resistant models

Since having categorized each of the SERM-resistant tumor models as Phase I or II resistant, we characterized the growth properties of cells which have been cultured long-term under estrogen-free conditions to determine whether resistance to estrogen withdrawal (as a surrogate for AI resistance) also evolves through distinct stages. Initially, we compared the proliferation of parental MCF-7 cells with two cell lines resistant to long-term estrogen withdrawal, MCF-7/ED (estrogen-deprived) and MCF-7/5C cells. MCF-7/ED cells were originally selected by culture of parental MCF-7 in estrogen-free medium for > 1 year, but were not cloned as a subline, rather they remain a population of cells. In a similar manner, MCF-7/5C cells were also derived from parental MCF-7 cells following long-term estrogen withdrawal, but were

cloned as a subline [56]. Notably, MCF-7/ED and MCF-7/5C cells were generated independently in different studies, that is, MCF-7/5C cells were not subcloned from the MCF-7/ED cells.

Growth of parental MCF-7, MCF-7/ED and MCF-7/5C cells was determined by measuring DNA amounts after 7 days in culture. Before beginning the experiment, parental MCF-7 cells were cultured for 4 days in estrogen-free media, since they had been maintained in fully-estrogenized medium. The experiment was started by seeding each of the cell lines in 24-well plates in estrogen-free medium. The cells were treated every 2 days with EtOH (vehicle control), 1 nM E₂, 10 nM FUL, and 1 nM E₂ plus 10 nM FUL. After 7 days, DNA quantities per well were determined using a fluorescence-based DNA assay. As expected in parental MCF-7 cells, E₂ induced growth by 6.9-fold (E₂ vs. control treatment), and this E₂-stimulated proliferation was completely blocked by the addition of FUL (E₂ + FUL vs. control) (Fig. 5). Hence, E₂ stimulated proliferation of parental MCF-7 cells in an ER-dependent manner.

Next, MCF-7/ED cells representing a population of cells resistant to estrogen withdrawal were characterized. MCF-7/ED cells grew maximally under estrogen-free conditions (control treatment, 100 % growth) and nearly maximally when treated with E₂ (80% of control) (Fig. 5). However, FUL and E₂ plus FUL treatment inhibited growth of MCF-7/ED cells (18% and 29%, respectively, of control) (Fig. 5). Thus, MCF-7/ED cell proliferation was largely unaffected by E₂, but dependence on the ER was demonstrated by the sensitivity of the cells to FUL.

Finally, we evaluated MCF-7/5C cells, which were a clonal derivative of long-term estrogen-withdrawn cells. MCF-7/5C cells grew maximally under estrogen-free conditions (control treatment, 100% growth), but E₂ treatment almost completely blocked proliferation (9% of control) (Fig. 5). Interestingly, FUL-treated MCF-7/5C cells exhibited significant growth (52% of control) (Fig. 5). Further, MCF-7/5C cells treated with E₂ plus FUL showed still greater amounts of proliferation (85% of control) (Fig. 5). In prior studies, we have demonstrated that MCF-7/5C cells undergo apoptosis when treated with E₂, and that co-treatment with FUL blocks this effect of E₂ [53]. Hence, MCF-7/5C cells required ER to be unliganded for maximal proliferation and survival, whereas E₂-bound ER led to cytostasis and apoptosis. Further, FUL reversed the apoptotic signal of E₂ and promoted proliferation.

We next verified that the MCF-7/5C cells behaved similarly *in vivo* as a xenograft tumor as they did *in vitro* in cell culture. MCF-7/5C cells were grown in culture and injected into 20 ovariectomized athymic mice. The animals were separated into 4 treatment groups of 5 mice each (10 tumors/group), corresponding to control (not treated), 0.3 cm E₂ capsule *sc*, 2 mg/day FUL *sc* (Faslodex), and 0.3 cm E₂ capsule *sc* plus 2 mg/day FUL *sc* (Faslodex). MCF-7/5C cells rapidly formed substantial tumors at every injection site (10 out of 10) in control-treated mice by 21 days after inoculation, but only 1 palpable tumor formed out of 10 injection sites in mice treated with E₂, resulting in a highly significant difference in the average tumor cross-sectional area between the two treatment groups (Fig. 6, $P < 0.0001$). In a prior report, we have shown that E₂ induces tumor regression and apoptosis in established MCF-7/5C xenograft tumors [53]. Importantly, MCF-7/5C xenograft tumors showed robust growth in the presence of FUL or E₂ plus FUL, which was not significantly different than growth of the control (no treatment) group, but was significantly greater than in the E₂ treatment group (Fig. 6, FUL vs. E₂, $P < 0.0001$; E₂+FUL vs. E₂, $P < 0.0001$). Hence, the MCF-7/5C xenograft tumor model was resistant to growth inhibition by FUL, and FUL treatment abrogated E₂-mediated growth inhibition.

Considering these varied growth responses together, parental MCF-7 cells model the therapeutic stage of antihormonal therapy; MCF-7/ED cells represent Phase I resistance to

estrogen withdrawal since they grew independent of E₂ yet remained sensitive to FUL; and MCF-7/5C tumors/cells were classified as Phase II resistant to estrogen withdrawal since E₂ inhibited their growth, but were resistant to growth inhibition by FUL or E₂ plus FUL.

3.6 Response of Phase II SERM-resistant tumor models to E₂ plus FUL (Fig. 7 and 8)

Since we observed that MCF-7/5C cells grew better when treated with E₂ plus FUL than with E₂ alone, we examined the effect of FUL in a background of physiologic E₂ in the Phase II SERM-resistant tumor models. The data from the MCF-7/RAL2 experiment depicted in Figure 3 was re-evaluated with an additional group of 5 animals (10 tumors) treated with a 0.3 cm E₂ capsule *sc* plus 2 mg/day FUL (Faslodex). MCF-7/RAL2 tumors treated with E₂ plus FUL showed robust growth compared to no palpable tumors in the E₂ alone ($P < 0.0001$), FUL alone ($P < 0.0001$), or control groups ($P < 0.0001$) (Fig. 7). Therefore, E₂ plus FUL, when combined, negated the growth inhibitory effects of either compound by itself.

We then tested whether this interaction between physiologic E₂ and FUL also occurred in the MCF-7/TAM2 tumor model of Phase II SERM resistance. However, this experiment was designed to evaluate effects of different treatments on tumors once they are established by allowing tumors to grow in the presence of TAM until they were palpable, and then randomized to different treatment groups. MCF-7/TAM2 tumor cores were implanted into 25 ovariectomized athymic mice. All animals were treated with 1.5 mg/day TAM *po* until tumors grew to an average cross-sectional area of 0.24 cm², at which time TAM treatment was withdrawn for 1 week to allow time for this drug to be completely metabolized and clear the animals' systems. Following the 1 week of TAM withdrawal, the average cross-sectional area of all tumors was 0.37 cm², and the animals were randomized into 5 groups of 5 mice each (10 tumors/group) corresponding to continuing 1.5 mg/day TAM *po*, 0.3 cm E₂ capsule *sc*, 2 mg/day FUL *sc*, 0.3 cm E₂ capsule *sc* plus 2 mg/day FUL *sc*, and control (no treatment). As would be predicted from the MCF-7/TAM2 experiment depicted in Figure 4, TAM treatment significantly stimulated growth ($P = 0.0026$) and E₂ significantly inhibited growth ($P = 0.0098$) compared to the control group on day 52 (Fig. 8). The size of FUL treated tumors was not significantly different than the control group. In contrast, we noted that tumors treated with the combination of E₂ + FUL did exhibit significantly greater growth than the control group (Fig. 8, $P = 0.018$). Thus, in a second model of Phase II SERM resistance, growth inhibition by E₂ alone was negated in the presence of FUL, leading to growth stimulation.

4. Discussion

We sought to discover unifying principles involved in the development of antihormone resistance by systematically studying the growth properties of a panel of antihormonally resistant MCF-7-based breast cancer xenograft tumor models. We have confirmed and extended prior observations [40–53] that have allowed the categorization of these tumor models as either Phase I or Phase II antihormone resistant. Phase I SERM resistance was characterized by growth stimulation in response to either a SERM or E₂ (MCF-7/RAL1 tumors, Figure 2), while in Phase II SERM resistance, only the SERM stimulated growth and E₂ inhibited growth (MCF-7/RAL2 and MCF-7/TAM2, Fig. 3 and 4, respectively). Phase I long-term estrogen withdrawn (AI) -resistant cells in culture grew independently of E₂ (MCF-7/ED cells, Figure 5), but Phase II resistant tumors were growth inhibited by E₂ (MCF-7/5C tumors, Figure 6). Hence, long-term blockade of ER activity by either SERMs or estrogen withdrawal can lead to selection of cells in which E₂ signals no longer proliferation, but rather inhibition, of growth, and as we have previously reported, apoptosis [48,51,53].

We also found that growth of Phase II SERM resistant tumors was not supported by FUL, but Phase II estrogen withdrawn-resistant tumors were cross-resistant to FUL (MCF-7/5C tumors, Fig. 6). Further, FUL combined with physiologic E₂ nullified the inhibitory effects of either

compound alone and led to stimulation of growth in Phase II SERM-resistant tumors (MCF-7/RAL2 and MCF-7/TAM2 tumors, Figures 7 and 8, respectively), and supported growth in Phase II estrogen withdrawn-resistant tumors (MCF-7/5C tumors, Fig. 6).

Noteworthy, the concentrations of FUL used in the cell culture proliferation experiments was 10 nM. We chose this concentration of FUL to reflect the circulating levels that are achieved clinically. In the clinic, FUL is not administered orally because of low bioavailability; rather it is given intra muscularly (as a single 250 mg dose once per month) to achieve slow constant release of the drug. In two independent multi-national Phase III clinical efficacy trials evaluating FUL in advanced breast cancer patients, the steady state circulating concentrations of FUL were determined to be approximately 6 – 7 µg/L (9.9 – 11.5 nM) in the European trial and 9 µg/L (14.8 nM) in the North American trial [57]. Hence, we used FUL at 10 nM in cell culture, reflecting the circulating concentrations of FUL achieved in women, but this concentration was much lower than the 100 nM to 1 µM FUL concentrations used in most cell culture studies. We hypothesize that the low circulating concentrations of FUL in patients may contribute to the lower than expected response rates in the clinic as would be predicted by the effectiveness of FUL in cell culture. In support of this hypothesis, we have found that while MCF-7/5C cells proliferate in the presence of 10 nM FUL, they do not in 1 µM FUL (unpublished, JS Lewis-Wambi and VC Jordan).

The distinct growth responses of the tumor and cell culture models studied here illustrate that resistance to hormonal blockade therapy continually evolves but can be separated into at least two phases (Fig. 9). Antihormonal resistance develops from selection of specific cell types that survive and proliferate when the ER is bound by a partial antiestrogen (Phase I SERM resistance) or unliganded (Phase I estrogen withdrawn-resistance). Prolonged hormonal blockade therapy maintains selective pressure, such that Phase I resistant cells continue to evolve to a Phase II resistant phenotype, and likely undefined additional phases. However, the study of Phase II antihormonal resistance has revealed a new biology of E₂ action involved in apoptosis that could be exploited to benefit breast cancer patients who have been exhaustively treated with SERMs and AIs. Moreover, the finding that FUL in a background of physiologic E₂ stimulated growth of Phase II resistant cells has important clinical implications. This knowledge can be implemented to optimize the application of third-line antihormonal therapy (Fig. 10). We propose that patients who have responded and then failed two antihormone therapies may exhibit Phase II resistant characteristics, and therefore respond to low-dose short-term estrogen therapy. The estrogen therapy would lead to apoptosis in the Phase II resistant cells and thereby debulk the tumor. Prior laboratory studies indicate that cells which remain are re-sensitized to first-line or second-line antihormonal therapy [47,49]. Hence, the low-dose short-term estrogen therapy would be followed by FUL plus an AI, to avoid the possible selection of cells that could grow in response to FUL plus physiologic E₂.

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Abbreviations

AI	aromatase inhibitor
ER	estrogen receptor
E₂	17 β -estradiol
FUL	fulvestrant
RAL	raloxifene
SERM	selective estrogen receptor modulator

Fig. 1.

Growth inhibition of MCF-7/E2 tumors in response to different FUL formulations and dosing schedules. Thirty ovariectomized athymic nude mice were bitransplanted in the axillary mammary fat pads with MCF-7/E2 tumor pieces 1 mm^3 in size. At the time of tumor implantation, the mice were separated into 6 treatment groups of 5 mice each, or 10 tumors per group. The treatment groups were Control (no treatment), 0.3 cm E₂ silastic capsule implanted *sc*, and 4 groups of different formulations/dosing schedules of 10 mg total FUL per week plus the 0.3 cm E₂ capsule *sc*. The 4 FUL formulations/dosing schedules corresponded to: 1) a 50 mg/ml suspension of FUL dissolved first in EtOH and then mixed with peanut oil, and administered 2 times per week as a 5 mg *sc* injection; 2) the clinically used Faslodex preparation consisting of a 50 mg/ml solution of FUL in EtOH and castor oil, and administered 5 times per week as a 2 mg *sc* injection; 3) a 50 mg/ml suspension of FUL dissolved first in DMSO and then mixed with peanut oil, and administered 2 times per week as a 5 mg *sc* injection; or 4) a 50 mg/ml solution of FUL in 100% DMSO, and administered daily 5 times per week as a 2 mg *sc* injection. Tumor growth was tracked by weekly measurements using Vernier calipers and calculating the tumor cross-sectional area according to the formula: $(\text{length}/2 \times \text{width}/2 \times \pi)$. The data shown represent the average tumor cross-sectional area (cm^2) per group $\pm$ SE. The cross-sectional area of E₂-treated tumors was statistically different from that of each of the four E₂ + FUL groups (all P -values < 0.0001). Also, the cross-sectional area of tumors in the E₂ + 5 mg FUL (EtOH/Peanut Oil suspension given 2 days per week) was statistically different from those in the E₂ + 5 mg FUL (DMSO/Peanut Oil suspension given 2 days per week) group ($P = 0.0013$). Likewise, the cross-sectional area of tumors in the E₂ + 2 mg FUL (EtOH/Castor Oil solution given 5 days per week) group was statistically

different from that of the E₂ + 2 mg FUL (100% DMSO solution given 5 days per week) group ($P = 0.0038$).

Fig. 2.

Growth stimulation of MCF-7/RAL1 tumors in response to E₂, and inhibition by FUL. Twenty ovariectomized athymic nude mice were implanted in the axillary mammary fat pads with 1 mm³ MCF-7/RAL1 tumor pieces and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day RAL *po*, 0.3 cm E₂ capsule *sc*, 2 mg/day FUL *sc*, and Control (no treatment). The data shown represent the average tumor cross-sectional area (cm²) per group $\pm$ SE. The cross-sectional area of RAL-treated ($P < 0.0001$) and E₂-treated MCF-7/RAL1 tumors ($P < 0.0001$) was significantly different from control-treated tumors.

Fig. 3.

Growth inhibition of MCF-7/RAL2 tumors in response to E₂ and FUL. Twenty ovariectomized athymic nude mice were bilaterally injected in the axillary mammary fat pads with 10⁷ MCF-7/RAL2 cells grown in culture and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day RAL *po*, 0.3 cm E₂ capsule *sc*, 2 mg/day FUL *sc*, and Control (no treatment). The data shown represent the average tumor cross-sectional area (cm²) per group ± SE. The cross-sectional area of RAL-treated MCF-7/RAL2 tumors was significantly different from E₂-treated, FUL-treated and Control-treated tumors (all *P*-values = < 0.0001).

Fig. 4.

Growth inhibition of MCF-7/TAM2 tumors in response to E₂ and FUL. Twenty ovariectomized athymic nude mice were implanted in the axillary mammary fat pads with 1 mm³ MCF-7/TAM2 tumor pieces and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day TAM *po*, 0.3 cm E₂ capsule *sc*, 2 mg/day FUL *sc*, and Control (no treatment). The data shown represent the average tumor cross-sectional area (cm²) per group $\pm$ SE. The cross-sectional area of TAM-treated ($P < 0.0001$) and E₂-treated MCF-7/TAM2 tumors ($P = 0.0004$) was significantly different from Control tumors ($P < 0.0001$).

Fig. 5.

Differential proliferation of MCF-7 long-term estrogen withdrawn cell culture models in response to E₂, FUL, and E₂ plus FUL for 7 days. Cells were cultured under estrogen-free conditions for 4 days, and then seeded at 2×10^4 cells per well in a 24-well plate. Beginning 24 hours after seeding (day 0) and every 2 days thereafter up to 6 days (days 2, 4, and 6), the cells were treated with 1 nM E₂, 10 nM FUL, 1 nM E₂ + 10 nM FUL, or Control (0.1% EtOH)-treated. The experiment was stopped on day 7. As a measure of proliferation, the amount of DNA per well was determined using a fluorescence-based DNA quantitation assay. Data are shown as the mean of 6 replicate wells per group $\pm$ SD. The experiment was performed 3 times independently, and one representative experiment is shown.

Fig. 6.

Growth inhibition of MCF-7/5C tumors in response to E₂ treatment, and resistance to FUL, and E₂ plus FUL. Twenty ovariectomized athymic nude mice were bilaterally injected in the axillary mammary fat pads with 10⁷ MCF-7/5C cells grown in culture and separated into 4 treatment groups of 5 mice each (10 tumors per group) corresponding to Control (no treatment), 0.3 cm E₂ capsule *sc*, 2 mg/day FUL *sc*, and 0.3 cm E₂ capsule *sc* + 2 mg/day FUL *sc*. The data are shown as a histogram on day 21 of the average tumor cross-sectional area (cm²) per group ± SE. The cross-sectional areas of Control-treated, FUL-treated, and E₂ plus FUL-treated MCF-7/5C tumors were each significantly different from E₂-treated (all *P*-values < 0.0001). However, the cross-sectional area of both FUL-treated and E₂ plus FUL-treated MCF-7/5C tumors were not significantly different from that of control-treated MCF-7/5C tumors.

Fig. 7.

E₂ plus FUL-stimulated growth of MCF-7/RAL2 tumors. Data are from Figure 3 on day 42 and shown as a histogram, but supplemented with the additional group of 5 ovariectomized athymic mice (10 tumors) treated with 0.3 cm E₂ capsule *sc* plus 2 mg/day FUL *sc*. The cross-sectional area of E₂ plus FUL-treated MCF-7/RAL2 tumors was significantly different from that of Control-treated, E₂-treated, and FUL-treated MCF-7/RAL2 tumors (all *P*-values < 0.0001).

Fig. 8.

E₂ plus FUL-stimulated growth of MCF-7/TAM2 tumors. Twenty-five ovariectomized athymic mice were implanted in the axillary mammary fat pads with 1 mm³ MCF-7/TAM2 tumor pieces, and then treated with 1.5 mg/day TAM *po* until the tumors were established at 0.24 cm², then TAM was withdrawn for 1 week. Following 1 week of TAM withdrawal, the tumors reached an average cross-sectional area of 0.37 cm² and the animals were separated into 5 treatment groups of 5 mice each (10 tumors per group) corresponding to 1.5 mg/day TAM *po*, 0.3 cm E₂ capsule *sc*, 2 mg/day FUL *sc*, 0.3 cm E₂ capsule *sc* plus 2 mg/day FUL *sc*, and Control (no treatment). The data shown represent the average tumor cross-sectional area (cm²) per group $\pm$ SE. The cross-sectional areas of MCF-7/TAM2 tumors at day 52 were compared by 1-way ANOVA. The cross-sectional areas of TAM-treated ($P = 0.0026$), E₂-treated ($P = 0.0098$), and E₂ plus FUL-treated MCF-7/TAM2 tumors ($P = 0.018$) were significantly different from control-treated tumors.

EVOLUTION OF RESISTANCE TO SERMs

EVOLUTION OF RESISTANCE TO ESTROGEN WITHDRAWAL

Fig. 9.

Evolution of antihormonal resistance in laboratory models of breast cancer. Long-term antihormonal therapy leads to selection of resistant cells that are stimulated to grow by a SERM or grow in an estrogen-depleted environment (AI resistance). Based on prior laboratory studies (4–10, 12, 13) and data presented here, the progression of antihormonal resistance can be separated into at least 2 phases defined by different growth responses to E₂ and FUL. In Phase I resistant disease, tumor cells are either growth stimulated by E₂ (as in SERM resistance) or grow independently of E₂ (as in estrogen withdrawn/AI resistance). However, these Phase I resistant cells remain dependent on ER since they are sensitive to growth inhibition by FUL. Selection of tumor cells continues during exhaustive antihormonal therapy until Phase II resistance develops, which is characterized by a new biology of E₂ action. Both Phase II SERM and estrogen withdrawn-resistant tumors respond to E₂ with growth inhibition and apoptosis. FUL still inhibits growth of Phase II SERM-resistant tumors, but not of Phase II estrogen withdrawn-resistant tumors. Moreover, FUL interacts with E₂ at physiologic concentrations to promote growth of both Phase II SERM and estrogen withdrawn-resistant disease. These emerging concepts on the evolution of antihormonal resistance based on laboratory studies have important implications for the utility of estrogen and FUL in the clinic.

TREATMENT PLAN FOR THIRD-LINE ANTIHORMONAL THERAPY

Fig. 10.

A proposed clinical strategy for the optimal application of third-line antihormonal therapy. We propose that patients who have initially responded to, and then failed, two previous antihormonal therapies may exhibit Phase II antihormonal resistance, and would then benefit from short-term low-dose estrogen, which would induce apoptosis in the Phase II resistant cells and debulk the tumor. Prior laboratory evidence indicates that the small percentage of Phase II tumors which revert to an estrogen-stimulated stage after estrogen-induced regression, are also re-sensitized to antihormonal therapy [47,49]. A low tumor burden would be maintained by FUL in an estrogen-depleted environment, *i.e.* FUL plus an aromatase inhibitor, to avoid the possible emergence of tumor growth through a negative interaction between FUL and physiologic estrogen.