Low-Molecular-Weight Cyclin E Can Bypass Letrozole-Induced G1 Arrest in Human Breast Cancer Cells and Tumors

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Abstract

**Purpose:** Low-molecular-weight cyclin E (LMW-E) in breast cancer cells induces genomic instability and resistance to inhibition by p21, p27, and fulvestrant therapy. Here, we sought to determine if LMW-E renders breast cancer cells unresponsive to aromatase inhibitors (AI), elucidate the mechanism of such resistance, and ascertain if inhibitors of LMW-E–associated kinase activity could overcome this resistance.

**Experimental Design:** The antiproliferative effects of the AIs were examined in aromatase-overexpressing MCF-7/Ac1 cells in the presence or absence of full-length cyclin E and LMW-E. Inhibition of LMW cyclin E kinase activity by roscovitine [a cyclin-dependent kinase (CDK) inhibitor] was examined in letrozole-unresponsive MCF-7/Ac1 cells. The role of LMW-E and CDK2 in mediating recurrence following AI treatment was also assessed in breast cancer patients.

**Results:** Overexpression of LMW-E in postmenopausal patients was associated with a poor prognosis. Letrozole, but not exemestane or anastrozole, mediated a pronounced G1 arrest in MCF-7/Ac1 cells. Androstenedione-induced G1 arrest correlated with increased cyclin E–associated kinase activity and increased CDK2 levels. Letrozole treatment inhibited cyclin E-CDK2 kinase activity by preventing the androstenedione-induced increase in CDK2. LMW-E bypassed this effect and rendered the cells resistant to letrozole inhibition. Roscovitine blocked the androstenedione-induced increase in CDK2, and LMW-E overexpression could not bypass this effect. Lastly, breast cancer patients whose tumors overexpress LMW-E were not responsive to AI treatment.

**Conclusions:** Roscovitine treatment can reverse intrinsic or acquired resistance to letrozole due to LMW-E expression in breast cancer cells. These data support the clinical investigation of CDK2 inhibitor therapy for postmenopausal women with estrogen receptor–positive, LMW-E–expressing breast cancer.

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is also associated with resistance to endocrine therapy and suggests that inhibitors of signal transduction pathways could provide additional treatment options. The neoadjuvant setting provides the opportunity to identify genes that differ in expression with response (or lack thereof) to treatment. For example, in a recent neoadjuvant study, increased expression of p44/p42 mitogen-activated protein kinase and HIF1α were independent predictors of resistance to letrozole (8). Taken together, these data suggest that identification and understanding of proteins that regulate response to AI treatment may provide critical information for the design of more effective treatment strategies.

Interest in cyclin E as a potential predictor of response to endocrine therapy originates from the associated cell cycle alterations of cyclin E, including decreased length of the G1 phase, more rapid transition of G1-S phase, increased cyclin E kinase activity, and increased genomic instability (9–12). The low-molecular-weight (LMW) forms of cyclin E are more likely to recur after AI treatment compared with those with low or no expression of LMW cyclin E. These data support clinical investigation of cyclin-dependent kinase 2 inhibitor therapy for postmenopausal women with estrogen receptor–positive, LMW cyclin E–expressing breast cancer.

### Translational Relevance

Although letrozole treatment of postmenopausal estrogen receptor–positive breast cancer reduces risk of early metastasis, resistance develops with time. Inhibition of cyclin E/cyclin-dependent kinase 2 activity through increased binding of the cell cycle inhibitor p27 to the complex is a key mediator of the anti-proliferative effects of letrozole. Overexpression of low-molecular-weight (LMW) cyclin E can bypass this process and renders letrozole ineffective in mediating growth arrest. Treatment of the cells with roscovitine overcomes the LMW cyclin E–mediated letrozole resistance. Lastly, we show that breast cancer patients whose tumors overexpress LMW cyclin E are more likely to recur after AI treatment compared with those with low or no expression of LMW cyclin E. These data support clinical investigation of cyclin-dependent kinase 2 inhibitor therapy for postmenopausal women with estrogen receptor–positive, LMW cyclin E–expressing breast cancer.

Inhibition by p21 and p27 (10). Taken together, the data about estrogens and their effect on the G1-S transition of the cell cycle suggest that aberrations at this checkpoint (i.e., cyclin E overexpression) may have a significant effect on the clinical benefit achieved from adjuvant endocrine therapy.

In this study, we set out to determine if the overexpression of LMW cyclin E leads to the lack of response to AI treatment in aromatase-overexpressing cells. We further examined the mechanism of this resistance and have proposed targeted therapy to overcome it. Lastly, we provide evidence that breast cancer patients whose tumors overexpress LMW cyclin E are not responsive to AI treatment.

### Materials and Methods

**Chemicals.** The AIs letrozole, exemestane, and anastrozole were provided by Astra Zeneca. These drugs were dissolved in methanol and diluted in tissue culture medium. Androstenedione was obtained from Sigma and the drug was diluted in ethanol. Vehicles (methanol or ethanol) alone were used as controls.

**Cell culture.** MCF-7/Ac1 was cultured in improved modified Eagle’s medium with 5% fetal bovine serum, 1% penicillin/streptomycin solution, and 600 μg/mL G418.

**Cell growth.** The effects of the AIs on MCF7/Ac1 cells growth were examined by counting the cells at the indicated time. The cells were detached from their flask using trypsin and counted using a Coulter counter machine.

**Cell cycle analysis.** MCF-7/Ac1 cells were cultured in estrogen-deprived media with or without 25 nmol/L 4-androstenedione, and were treated with different concentrations of AIs. Nontreated and drug-treated cells were collected 72 h later for flow cytometry and lysates were prepared for Western blot and kinase assays.

**Flow cytometry analysis.** Cells were pelleted and resuspended in 1.5 mL of PBS and then fixed in 3.5 mL of 95% ethanol overnight at −20°C. After being washed, the pellets were resuspended in a solution of PBS containing 10 μg/mL propidium iodide, 20 μg/mL RNase A, 0.5% Tween 20, and 0.5% bovine serum albumin, and were incubated at 37°C for 30 min. The profiles of cells in the G0-G1, S, and G2-M phases of the cell cycle were analyzed at the M.D. Anderson Cancer Center Cytometry Core Facility on a FACSCalibur machine equipped with Cellquest or ModFit software.

**Western blot analysis.** Cell lysates were prepared and subjected to Western blot analysis as previously described (10). Briefly, 50 μg of protein were subjected to electrophoresis on SDS-PAGE and transferred to Immobilon P overnight at 4°C at 35 V constant voltage. The blots were blocked overnight at 4°C in BLOTTO [5% nonfat dried milk in 20 mmol/L Tris, 137 mmol/L NaCl, and 0.05% Tween (pH 7.6)]. After being washed, the blots were incubated in primary antibodies for 3 h. Primary antibodies used were cyclin E (HE-12; Santa Cruz Biotechnology), p21 (OP64; Oncogene Research Products), p27 (K25020; BD Biosciences-Transduction Laboratories), CDK2 (Transduction Laboratories), and actin (Chemicon International, Inc.). Blots were then incubated with goat
anti-mouse or anti-rabbit immunoglobulin–horseradish peroxidase conjugate at a dilution of 1:5,000 in BLOTTO for 1 h and finally washed and developed by using the Renaissance chemiluminescence system as directed by the manufacturer (Perkin-Elmer Life Sciences, Inc.). Western blots were quantitated by densitometric analysis using the IPLab Gel software (Scientific Image Processing). Densitometric values of actin were used to standardize for equal protein loading. These values were introduced into the software GraphPad Prism version 4.0 (GraphPad Software, Inc.) for statistical analysis.

**Immunoprecipitation and immunoblotting.** Two hundred fifty micrograms of cell extracts were used per immunoprecipitation with polyclonal antibody to cyclin E or polyclonal antibody to CDK2, coupled to protein A beads. After being washed, the immunoprecipitates were subjected to electrophoresis in 13% gels, transferred to Immobolin P, blocked, and incubated with the indicated antibodies as already described.

**Protein kinase assays.** For histone H1 kinase assays, the immunoprecipitates were incubated with kinase assay buffer containing 60 μmol/L cold ATP, 5 μCi of [32P] ATP, and 5 μg of histone H1 (Roche Diagnostics Corp.) in a final volume of 30 μL at 37°C for 30 min. The products of the reaction were analyzed on 13% SDS-PAGE gels, and the gels were stained, destained, dried, and exposed to X-ray film. For quantitation, the protein bands corresponding to histone H1 were excised, and the radioactivity of each band was measured by Cerenkov counting.

**Study patients.** The clinical and pathologic data from 395 breast cancer patients, 390 of whom had data available regarding ER status, were previously reported by Keyomarsi et al. (16). Another group of patients included 128 women treated for breast cancer at M.D. Anderson Cancer Center with AIs (121 with anastrozole, 4 with letrozole, 2 with exemestane, and 1 with letrozole followed by exemestane) between 2001 and 2009. This group of AI-treated women was selected from patients cohort.

![Image](https://example.com/image.png)

**Fig. 1.** Relationship between ER status and cyclin E in breast cancer. Breast cancer specimens from 395 patients were assessed for ER and cyclin E expression. A, OS was higher in patients with ER-positive (n = 234) tumors (P = 0.0029). B, patients with ER-positive tumors were stratified by LMW cyclin E expression. Patients with high LMW cyclin E had markedly decreased OS (median OS, 3.25 y) compared with those with low levels of LMW cyclin E (median OS not reached; P < 0.0001). C, the relationship between OS, ER, and LMW cyclin E was maintained in postmenopausal patients (P < 0.001).
enrolled in an Institutional Review Board–approved protocol to study the cyclin E deregulation in breast cancer. From all the patients enrolled in this study at the time of surgery, freshly resected breast cancer tissue samples were collected and subjected to protein extraction and Western blot analysis of cyclin E expression. Demographic, clinical, and pathologic data including the steroid-receptor status as well as the low molecular cyclin E levels are described in Supplementary Table S1.

**Statistical analysis.** Overall survival (OS) was calculated from the date of surgical excision of the primary tumor to the date of death or last follow-up. OS survival curves were computed by the Kaplan-Meier method (17). Univariate analyses of OS survival according to levels of ER and LMW cyclin E were done with the use of a two-sided log-rank test (18). Results are shown as mean ± SD. Differences were considered significant when the two-tailed Student's t test showed differences at P < 0.05.

**Results**

**Overexpression of LMW cyclin E in postmenopausal breast cancer patients is indicative of a poor prognosis irrespective of ER status.** In a retrospective study of 395 patients, we have previously reported on the strong prognostic value of cyclin E in breast cancer (16). We have recently reanalyzed the data to determine the relevance of LMW cyclin E as a prognostic factor based on the ER status of the tumor (Fig. 1). The 5-year OS rates were significantly higher in ER-positive patients compared with ER-negative patients (P = 0.0029; Fig. 1A). We next stratified the 234 ER-positive patients as a function of LMW cyclin E expression and found that those patients who had ER-positive tumors who also had high levels of LMW cyclin E had worse outcome compared with those patients whose tumors were ER positive and had low levels of LMW cyclin E (P < 0.0001; Fig. 1B). This relationship held when only the postmenopausal patients were included in the analysis (P < 0.0001; Fig. 1C). Given this relationship between LMW cyclin E and ER status in this cohort of breast cancer patients, we sought to investigate whether LMW cyclin E may effect responsiveness to hormonal therapy. We specifically chose to investigate the effect on responsiveness to AIs, which currently are the standard of care for postmenopausal patients with HR-positive breast cancer.

**Effect of AI treatment on proliferative response and the cell cycle distribution of MCF-7/Ac1 cells.** We used MCF-7/Ac1 cells, which are MCF7 breast cancer cells that have been transfected with the gene for aromatase, the enzyme responsible for the conversion of androgens to estrogens. These cells can be stimulated to grow using the aromatizable androgen, androstenedione, which is transformed into estrogen by the aromatase activity of the cells as previously reported (19). This model system simulates the postmenopausal breast cancer patient.

To examine the effects of the three different AIs on the proliferation of MCF-7/Ac1 cells, cells were cultured in estrogen-deprived, charcoal-stripped serum media (CSSM) for 4 days before treatment. The response of the cells to androstenedione alone or androstenedione plus one of the three AIs, letrozole (Fig. 2A), anastrozole (Fig. 2B), and exemestane (Fig. 2C), was measured after 3 days of treatment. Cells maintained in CSSM were used as controls. Compared with control cells (CSSM), 25 nmol/L androstenedione treatment resulted in a 3.9- ± 1.3-fold increase in cell number. The androstenedione-induced growth of MCF-7/Ac1 cells was inhibited by letrozole by 37.2% ± 10.4% at 0.1 μmol/L and 63.5% ± 13.8% at 1 μmol/L (Fig. 2A). The antiproliferative effect of letrozole is comparable with that in cells cultured in estrogen-deprived media (CSSM). Anastrozole did not inhibit the androstenedione-induced growth of MCF-7/Ac1 cells at any of the concentrations tested, whereas exemestane partially inhibited their growth by 25% ± 3% at 1 μmol/L and 42% ± 8% at 10 μmol/L (P < 0.05 versus nontreated cells). These results show that MCF-7/Ac1 cells are more responsive to letrozole than to exemestane or anastrozole.

To investigate the causes of the antiproliferative effects of AIs, cells were stained with propidium iodide and cell cycle analysis was done by flow cytometry (Fig. 2, right). Androstenedione treatment increased the fraction of cells in S phase by 7.3-fold compared with vehicle-treated cells (40.3% ± 1.9% versus 5.5% ± 1.7%) with a concomitant decrease in G0–G1. Among all the treatments, letrozole caused the greatest accumulation of cells (61.2% ± 1.5%) in G0–G1 compared with control cells (72.1% ± 1.9%) and a significant decrease in the number of cells in S phase (13.1% ± 2.3%) compared with control cells (5.5% ± 1.7%). Exemestane at 10 μmol/L caused an increase in G0–G1 cells from 35.1% ± 0.8% to 49.2% ± 0.7% with a decrease in the number of cells in S phase from 40.3% ± 1.9% to 31.7% ± 2.5%, whereas anastrozole at 10 μmol/L had a more subtle effect. The flow cytometry data correlate with the effect observed on cell number with letrozole having the strongest antiproliferative effects on MCF-7/Ac1 cells due to the disruption of cell cycle progression by causing growth arrest at the G1 phase of the cell cycle.

**Mechanism of letrozole induced G1 arrest.** After observing the induction of G1 arrest by AIs, we set out to examine the mechanism involved. Because letrozole was the most effective of the three inhibitors, we tested this drug in subsequent experiments. We investigated the effect of increasing concentrations of letrozole on the cyclin E–associated kinase activity (Fig. 3A) and on the CDK2-associated kinase activity (Supplementary Fig. S1). Androstenedione treatment increased the cyclin E–associated kinase activity by 1.6-fold and the CDK2-associated kinase activity by 2.6- to 3.5-fold compared with vehicle-treated cells. Letrozole blocked this increase in cyclin E– and CDK2-associated kinase activity at a concentration as low as 0.125 μmol/L (Supplementary Fig. S1; Fig. 3A). Western blot analysis showed that androstenedione treatment increased the CDK2 protein levels by 2.3-fold when compared with vehicle-treated cells, whereas letrozole treatment blocked
the increase in CDK2 protein levels in a dose-dependent manner (Fig. 3B). Active CDK2 is depicted by an increase in phospho-CDK2 band shown both in the Western blot analysis using the pan-CDK2 antibody or using a phospho-specific CDK2 antibody, which increased by 3.5-fold in androstenedione-treated cells. We also show that increasing the concentration of letrozole leads to a block of androstenedione-induced increase in CDK2 kinase activity.

**Fig. 2.** Effect of AI treatment on proliferative response and cell cycle distribution of MCF-7/Ac1 cells. Cells were cultured in improved modified Eagle’s medium (IMEM) with 10% CSSM without phenol red and with 600 μg/mL of G418 for 4 d before plating. Cells (100,000) were seeded in 100-mm dishes and, 24 h later, were exposed for 3 d to the specific treatment. A, antiproliferative effect of increasing concentrations of letrozole in the presence of 25 nmol/L of androstenedione (AD) on MCF-7/Ac1 cell growth (left) and cell cycle distribution (right). Cell growth is expressed as the percentage of the cells compared with the control cells (25 nmol/L androstenedione-treated cells; 575,000 cells at day 3). Columns, mean of two to three experiments, each in triplicates; bars, SD; *, P < 0.05, when compared with cells only treated with 25 nmol/L androstenedione; n.s, not significant. CSSM, nontreated cells cultured in CSSM without phenol red and with 600 μg/mL of G418. B, antiproliferative effect of increasing concentrations of anastrozole in the presence of 25 nmol/L of androstenedione on MCF-7/Ac1 cell growth (left) and cell cycle distribution (right). C, antiproliferative effect of increasing concentrations of exemestane in the presence of 25 nmol/L of androstenedione on MCF-7/Ac1 cell growth (left) and cell cycle distribution (right).
that parallel decreased phospho-T160-CDK2 (Supplementary Fig. S1). Additionally, letrozole treatment also results in the decreased phosphorylation of the endogenous CDK2 substrate, pRb (Supplementary Fig. S1). Cyclin E protein levels were not affected by androstenedione treatment and slightly decreased at 0.5 μmol/L letrozole. P27 protein levels remained stable and were independent of drug treatments. To define the molecular basis of the cyclin E and CDK2 kinase inhibition, we performed immunoprecipitation with cyclin E (Fig. 3C) and CDK2 (Fig. 3D) antibodies followed by Western blot analysis (50 μg of protein) with the indicated antibodies. C, letrozole induced increased binding of p27 to cyclin E complexes. The same cell lysates were first immunoprecipitated with cyclin E followed by Western blot for p21 and p27. D, letrozole induced increased binding of p27 to CDK2 complexes. The same cell lysates were first immunoprecipitated with CDK2 followed by Western blot for p21 and p27. The levels of proteins were measured by densitometric scanning of the corresponding bands and normalized using actin values. The values indicated at the bottom were compared with the values obtained with nontreated cells.

Fig. 3. In vitro antiproliferative effect of increasing concentrations of letrozole in the presence of 25 nmol/L androstenedione (AD) on MCF-7/Ac1 human breast cancer cells. A, letrozole blocks the androstenedione-induced increase in cyclin E–associated kinase activity in MCF-7/Ac1 cells. MCF-7/Ac1 cells were treated with the indicated concentrations of letrozole and androstenedione for 3 d. Cyclin E kinase assays were done by immunoprecipitating equal amounts of cell lysate (250 μg) with monoclonal antibodies to cyclin E coupled to protein A beads, using histone H1 as substrate. B, letrozole blocks the androstenedione-induced increase in CDK2 protein levels. The same cell lysates were subjected to Western blot analysis (50 μg of protein) with the indicated antibodies.
interrogated the role of full-length and LMW cyclin E in letrozole response. To this end, we examined the sensitivity of cyclin E-overexpressing MCF-7/Ac1 cells to the growth-inhibitory effect of letrozole using adenoviruses to overexpress full-length and LMW cyclin E (Fig. 4). MCF-7/Ac1 cells were cultured in CSSM for 4 days before infecting them with 4,000 multiplicity of infection (m.o.i.) of either LacZ, full-length cyclin E (cyclin EL), or LMW cyclin E (cyclin E-T1 and cyclin E-T2). Twenty-four hours later, cells were left either nontreated, treated with 25 nmol/L androstenedione alone, or treated with 25 nmol/L androstenedione plus 1 μmol/L letrozole for an additional 3 days (Fig. 4A and B). Following the treatment, cells were enumerated or subjected to flow cytometry analysis. The results revealed that the androstenedione-induced growth of MCF-7/Ac1 cells was inhibited by letrozole by 40.8% (P = 0.029) in uninfected cells and by 56.1% (P < 0.01) in LacZ-infected cells, whereas no significant growth inhibition was observed in cyclin EL–, T1–, and T2–infected cells (P > 0.05; Fig. 4A). However, flow cytometric analysis revealed that letrozole treatment caused a significant decrease in the number of cells in the S phase in uninfected (62%), LacZ-infected cells (37%), and cyclin EL–infected cells (42%), whereas cyclin E-T1– and T2–infected cells were partially resistant to letrozole-induced decrease in the S-phase fraction (16% and 21%, P < 0.01 versus cyclin EL; Fig. 4B). These results show that although cyclin E overexpression could override the letrozole inhibition of androstenedione-induced increase in cell number (Fig. 4A), only the LMW cyclin E–overexpressing cells could override the letrozole inhibition of androstenedione-induced S-phase entry (Fig. 4B).

To determine if cyclin E overexpression could rescue the block by letrozole of androstenedione-induced CDK2 protein levels, the same samples were used to determine the cyclin E and CDK2 protein levels by Western blot analysis. The exogenous forms of cyclin E were expressed at 2- to 4-fold higher levels than endogenous cyclin E, and the Western blot in Fig. 4C shows that the cyclin E protein levels were not affected by the drug treatments. We also show that the LMW cyclin E protein levels achieved by adenoviral expression is comparable with the levels seen in human breast tumor samples (Supplementary Fig. S2). Androstenedione treatment induced a 1.8-fold increase in total CDK2 protein levels in uninfected and LacZ-infected cells, whereas letrozole treatment downregulated the total CDK2 protein levels to 10% of the level in uninfected cells. Letrozole treatment of cyclin EL–overexpressing cells downregulated the total CDK2 protein levels to only 50% of the level found in nontreated, uninfected cells. In sharp contrast, in nontreated, LMW cyclin E–overexpressing cells, the CDK2 protein levels were already 3.6-fold (for cyclin E-T1) and 1.6-fold (for cyclin E-T2) higher than in nontreated uninfected cells and did not drop following letrozole treatment. Densitometric scanning of the Western blots revealed a 1.5- to 2-fold increase in the amount of phosphorylated, active CDK2 bands (bottom band) in LMW cyclin E–overexpressing cells compared with cyclin EL–overexpressing cells, consistent with higher CDK2 kinase activity that is resistant to letrozole inhibition (Fig. 4C, bar graph). Furthermore, increasing concentrations of the cyclin E-T1 virus increase the CDK2 kinase activity in a dose-dependent manner, 1.8– to 5-fold at 500 m.o.i., 5– to 9.7-fold at 1,000 m.o.i., and 5.8– to 14.4-fold at 4,000 m.o.i. when compared with the CDK2 kinase activity in androstenedione-treated LacZ cells (Supplementary Fig. S3). Lastly, we show that 1 μmol/L letrozole treatment of LMW cyclin E (T1)–expressing cells cannot block the CDK2 kinase activity at any of the cyclin E-T1 adenovirus m.o.i., whereas in LacZ-expressing cells, letrozole completely blocks the androstenedione-induced increase in CDK2 kinase activity (Supplementary Fig. S3). These results show that cyclin E overexpression can prevent the block by letrozole of androstenedione-induced CDK2 partially for cyclin EL and completely for cyclin E-T1 and cyclin E-T2–overexpressing cells. Cyclin E-T1–overexpressing cells maintain a high CDK2 kinase activity that is insensitive to letrozole inhibition (Supplementary Fig. S3).

LMW cyclin E–overexpressing MCF-7/Ac1 cells cannot bypass the block by roscovitine of androstenedione-induced increase in cell number. Because our results thus far showed that androstenedione and LMW cyclin E cell proliferation and that G1 exit is mediated by increased CDK2 protein levels and activity, we questioned if a CDK inhibitor such as roscovitine could block this effect. To directly address this question, MCF-7/Ac1 cells were cultured in CSSM for 4 days before adding medium with no virus or with 4,000 m.o.i. of either LacZ or LMW cyclin E (cyclin E-T1) adenoviruses. Twenty-four hours later, cells were left nontreated, treated with 25 nmol/L androstenedione alone, or treated with 25 nmol/L androstenedione plus 1 μmol/L letrozole for 3 days (Fig. 4D). The androstenedione-induced growth of MCF-7/Ac1 cells was inhibited by letrozole by 51% in uninfected cells and by 58% in LacZ-infected cells, whereas the growth of cyclin E-T1–infected cells was inhibited by only 20% (Fig. 4D, left). In sharp contrast, 20 μmol/L of roscovitine completely inhibited the androstenedione-induced increase in cell number in uninfected, LacZ–, or cyclin E-T1–infected cells (Fig. 4D, right). These results show that LMW cyclin E–overexpressing MCF-7/Ac1 cells cannot bypass the block by roscovitine of androstenedione-induced increase in cell number.

Roscovitine blocks the androstenedione-induced increase in active (phosphorylated) CDK2, and LMW cyclin E overexpression cannot bypass this effect. We next examined if roscovitine could also block the growth of letrozole-resistant LMW cyclin E–overexpressing MCF-7/Ac1 cells. To this end, cyclin E-T1–infected (i.e., LMW) MCF-7/Ac1 cells were sequentially treated with androstenedione in the presence or absence of letrozole for 3 days, followed by 20 μmol/L of roscovitine for an additional 2 days (Fig. 5). Medium alone or medium plus DMSO were used as controls. A schematic of the treatment strategy is depicted in Fig. 5A. At the conclusion of the treatment, cells were enumerated and subjected to Western blot analysis with CDK2 (total) and phospho-CDK2 antibodies. The results revealed that
Fig. 4. LMW but not full-length cyclin E–overexpressing MCF-7/Ac1 cells could partially override the letrozole inhibition of androstenedione (AD)-induced G1 exit and androstenedione-induced CDK2 protein levels. A, cyclin E overexpression overrides the letrozole inhibition of androstenedione-induced proliferation. MCF-7/Ac1 cells were cultured in IMEM with 10% CSSM without phenol red and with 600 μg/mL of G418 for 4 d before plating. Triplicate wells of six-well plates were then infected with the indicated adenoviruses (at 4,000 m.o.i.) 24 h before drug treatment. Cells were then left nontreated (E2W, estrogen withdrawal) or treated with 25 nmol/L androstenedione, or treated with 25 nmol/L androstenedione and 1 μmol/L letrozole (AD + Let) and collected 3 d later for cell number. Cell growth is expressed as the percentage of the cells compared with the control cells (25 nmol/L androstenedione-treated cells). B, LMW cyclin E–overexpressing MCF-7/Ac1 cells could partially override the letrozole inhibition of androstenedione-induced G1 exit. MCF-7/Ac1 cells were treated as described in A and were collected for flow cytometry analysis. Histograms represent the S-phase fraction expressed as the percentage of the cells in S phase compared with the control cells (25 nmol/L androstenedione-treated cells). C, cyclin E overexpression prevented the block by letrozole of androstenedione-induced CDK2 protein levels. The same cell lysates as in A and B were subjected to Western blot analysis (50 μg of protein) with cyclin E and CDK2 antibodies. Bar graph, the densitometric values of the phosphorylated CDK2 bands. D, LMW cyclin E–overexpressing MCF-7/Ac1 cells cannot bypass the block by roscovitine of androstenedione-induced increase in cell number. Left, MCF-7/Ac1 cells were cultured in IMEM with 10% CSSM without phenol red and with 600 μg/mL of G418 for 4 d before plating. Cells were then infected with the indicated adenoviruses (at 4,000 m.o.i.) 24 h before drug treatment. Cells were then treated with 25 nmol/L androstenedione and 1 μmol/L letrozole, and collected 3 d later for cell number. Right, cells were treated as in A except that letrozole was replaced by 20 μmol/L of roscovitine. Columns, mean of two to three experiments, each in triplicates; bars, SD.
Fig. 5. Roscovitine blocks the androstenedione-induced increase in active (phosphorylated) CDK2, and LMW cyclin E overexpression cannot bypass this effect. A, schematic representation of the experimental design. B, MCF-7/Ac1 cells were cultured in IMEM with 10% CSSM without phenol red and with 600 μg/mL of G418 for 4 d before plating at a density of 100,000 cells for a 100-mm dish. Cells were then infected with the indicated adenoviruses (at 4,000 m.o.i.) 24 h before drug treatment. Cells were then treated with 25 nmol/L androstenedione and 1 μmol/L letrozole and collected 3 d later for cell number (3d) followed by 2 d in medium (CSSM) alone (3d + 2d) or medium (CSSM) plus DMSO (3d + 2d DMSO) or medium (CSSM) plus 20 μmol/L of roscovitine (3d + 2d Rosco). C, the same cells used for counting were collected and lysates were subjected to Western blot analysis (25 μg of protein) with either the CDK2 (D-12) or phospho-T160-CDK2 antibody. D, top, ratio of densitometric values of phosphorylated CDK2/total CDK2. Bottom, densitometric values of the phospho-T160-CDK2 bands in androstenedione + letrozole–treated cells.
3 days of letrozole treatment blocked the androstenedione-induced increase in cell number in uninfected and LacZ-infected MCF-7/Ac1 cells, whereas LMW cyclin E–overexpressing cells were resistant to letrozole inhibition (Fig. 5B). Culturing of cells for an additional 2 days in CSSM or medium plus DMSO led to a 4- and 3.5-fold increase in androstenedione-induced proliferation for uninfected and LacZ-infected cells, respectively, and a 2.3- and 2.8-fold increase in androstenedione-induced proliferation for LMW cyclin E–overexpressing cells. This androstenedione-induced proliferation was blocked by roscovitine concomitant with the disappearance or decrease in phosphorylated, active CDK2 protein as shown by Western blot analysis (Fig. 5C, bottom band). In LMW cyclin E–overexpressing cells, letrozole treatment did not prevent a 2.5- and 2.8-fold increase in androstenedione-induced proliferation nor did it decrease the phosphorylated/unphosphorylated CDK2 ratio (134–234%; Fig. 5D, top). On the other hand, treatment of cells with 20 μmol/L of roscovitine was sufficient to completely block the proliferation of letrozole-resistant cells concomitant with a decrease in phosphorylated/unphosphorylated CDK2 ratio to 69% (Fig. 5D, bottom). These results show that roscovitine blocks the androstenedione-induced increase in active (phosphorylated) CDK2, and LMW cyclin E overexpression cannot bypass this effect. These results also suggest that roscovitine treatment of breast cancer cells can reverse intrinsic or acquired resistance to letrozole as a result of LMW cyclin E expression.

**Increased risk of recurrence in AI-treated patients with high LMW levels in primary tumors.** To determine the relationship between levels of LMW cyclin E in breast cancer tissues and resistance to AI treatment, we performed an analysis of recurrence rate in 128 AI-treated breast cancer patients with high (28 of 128) and low (100 of 128) LMW levels in primary tumors. (Supplementary Table S1; Fig. 6A). Patient demographics are depicted in Supplementary Table S1. We found that AI-treated patients with high LMW tumors have increased frequency of recurrence (4 of 28, 14.3%) when compared with patients with low LMW tumors (3 of 100, 3.0%; Fisher's exact test \( P = 0.041 \)). B, Western blot analysis to measure CDK2 protein levels in breast cancer tissues from patients with high LMW cyclin E levels who did not relapse \((n = 4)\), from patients with high LMW cyclin E levels who relapsed \((n = 4)\), from patients with low LMW cyclin E levels who did not relapse \((n = 3)\), and from patients with low LMW cyclin E levels who relapsed \((n = 3)\). Lysates were subjected to Western blot analysis (50 μg of protein) with CDK2 (D-12, sc-6248). Total cyclin E levels were determined by Western blot analysis and densitometry was used to quantitate full-length and LMW forms for each sample. The densitometric values of LMW cyclin E are presented in the bar graph. Units used are arbitrary. C, AI-resistant tumors have increased levels of CDK2. Six of seven patients with recurrent disease had increased CDK2 protein levels compared with one of seven patients with no relapse \((P = 0.0291, \text{Fisher's exact test})\).
breast cancer samples from patients being disease free after AI treatment at the time of the last contact (n = 7; Fig. 6B). These results revealed that six of seven patients with recurrent disease had increased CDK2 protein levels compared with one of seven patients with no relapse (P = 0.0291, Fisher’s exact test; Fig. 6C). Among the high LMW cyclin E group, four of four AI-resistant patients (who had relapse) had increased CDK2 protein levels compared with one of four patients with no relapse (P = 0.1429, Fisher’s exact test). These results suggest that the overexpression of LMW cyclin E and increased CDK2 protein levels not only can predict potential AI treatment failure but also provide a rational basis of treatment of these patients with CDK inhibitors.

Discussion

In this report, we show that overexpression of the LMW forms of cyclin E render letrozole therapy ineffective in breast cancer cells that express both aromatase and ER. The mechanism of this effect is through the LMW cyclin E–mediated induction of the CDK2 activity. When LMW cyclin E is present, it results in higher CDK2 activity and resistance to p21 and p27 inhibition. Treatment of cells with letrozole leads to increased binding of p27 to CDK2, resulting in the inactivation of CDK2. An event such as overexpression of LMW cyclin E, which can bypass this process, will render letrozole ineffective in mediating a growth arrest in these cells. We also show that treatment of cells with roscovitine can overcome this LMW cyclin E–mediated letrozole resistance. As such, our data provide an alternative treatment option for those postmenopausal breast cancer patients whose tumors are ER positive but express the resistance to letrozole. As such, our data provide an alternative treatment option for those postmenopausal breast cancer patients whose tumors are ER positive but express the resistance to letrozole.

A major issue in the treatment of hormone receptor–positive breast cancer is resistance to endocrine therapy. This resistance is intrinsic in up to 50% of patients and acquired in all patients with metastatic disease. Mechanisms of resistance to letrozole include a genetic polymorphism in the aromatase gene CYP19 (20), high levels of ER expression driving transcription (21), or a constitutively active ER that does not require estrogen for activation (22). Cancer cells can also acquire resistance to letrozole by activation of the HER-2/mitogen-activated protein kinase pathway, and in these cases, trastuzumab plus letrozole is more effective than either drug alone in letrozole-refractory tumors (23). Other growth factor pathways, including insulin-like growth factor receptor and the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin pathways, have been shown to play a role in the resistance to endocrine agents, and combination treatments targeting multiple pathways are more effective (24, 25).

Here, we show that the activation of CDK2 by overexpression of the LMW forms of cyclin E is a novel mode of letrozole resistance, one that can be circumvented with CDK inhibitors.

Cyclin E protein is overexpressed and posttranslationally cleaved by elastase into LMW isoforms (26). LMW cyclin E accumulation is tumor specific and these isoforms have been found in multiple tumor types including breast, ovarian and colorectal cancers, and melanomas (27–31). Furthermore, LMW cyclin E proteins are strong correlative biomarkers in breast and ovarian cancers (16, 30). The LMW cyclin E isoforms have a more profound effect on cell cycle deregulation than the full-length cyclin E (EL) protein (10, 26, 28, 29, 32, 33), and transgenic mice expressing the LMW cyclin E isoforms have more mammary tumor development and metastasis than transgenic mice with the full-length cyclin E (EL; ref. 34). Thus, the LMW cyclin E isoforms seem more aggressive than EL in cell cycle abrogation and mammary tumor initiation and maintenance. Cyclin E has also been implicated in antiestrogen resistance. A study found that the association between cyclin E and disease outcome was restricted to patients who were treated with tamoxifen in the adjuvant setting (15). Another study using MCF-7 cells reported that overexpression of cyclin E could counteract tamoxifen-mediated growth arrest in human breast cancer patients (35). Our laboratory has previously shown that overexpression of LMW cyclin E in breast cancer cells is associated with resistance to fulvestrant (10). Here, we describe a novel mechanism of letrozole resistance through the overexpression of LMW cyclin E leading to sustained activation of CDK2. Patients with high LMW cyclin E levels and ER-positive tumors would likely not respond to letrozole treatment but could benefit from a therapy targeting the cyclin E/CDK2 complexes such as roscovitine (Seliciclib or CYC202).

Until now, the use of CDK inhibitors in human malignancies has been of limited success. This may be due to the suboptimal selection of the group of patients that would benefit the most from the therapy. We show in our model system that the conversion of androstenedione into estrogen by the aromatase enzyme activity strongly stimulates the growth of breast cancer cells by increasing the CDK2 kinase activity leading to increase in the S-phase fraction. Our study shows that letrozole treatment blocks the androstenedione-induced increase in S-phase fraction, which would be translated to a low Ki67 labeling index in a responding tumor. The Ki67 labeling index before and after neoadjuvant endocrine therapy could identify the nonresponding ER-positive, LMW cyclin E–positive tumors that could benefit from a CDK2-targeted therapy. Additionally, this study suggests that there is a need to identify the population of patients that may benefit from CDK inhibitors (i.e., overcoming the weaknesses of prior studies that were limited by poor patient selection) and that our data suggest that tumors from patients with ER-positive disease should be assessed for expression of LMW cyclin E in an effort to predict who may respond to letrozole and who could also benefit from CDK2-targeted therapy.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Low-Molecular-Weight Cyclin E Can Bypass Letrozole-Induced G₁ Arrest in Human Breast Cancer Cells and Tumors

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