Cancer Therapy: Preclinical

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 exit 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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Endocrine therapy is an important part of the management of patients with hormone receptor–positive breast cancer. Approximately 75% of postmenopausal women with breast cancer have tumors that express the estrogen receptor (ER) and/or progesterone receptor, suggesting that they may benefit from such targeted therapy. These patients will routinely be offered a third generation aromatase inhibitor (AI) such as anastrozole, exemestane, or letrozole. These agents have been shown to be well tolerated and their use results in improved disease-free survival compared with the selective ER modulator, tamoxifen, when used in the adjuvant setting (1–3). Letrozole has also been shown to result in greater reduction in tumor size and increased utilization of breast conserving surgery when compared with tamoxifen in the neoadjuvant setting (4).

Despite the effectiveness of AIs, not all patients respond to this treatment, and in those who do, resistance develops after prolonged exposure. In a recent study, the value of proliferation as measured by Ki67 in predicting response to AIs was evaluated. This randomized, double-blind, phase III study showed that letrozole improved disease-free survival compared with tamoxifen for postmenopausal women with hormone receptor–positive disease (1, 5). The investigators found a greater benefit from letrozole compared with tamoxifen in tumors with a higher Ki67 labeling index, suggesting that high Ki67 labeling index levels may identify a patient group that could benefit from letrozole as their initial adjuvant therapy (6). With respect to resistance to AI therapy, in the majority of cases, ERα expression is not lost (7); however, there are alterations in downstream signaling genes and proteins. Increased growth factor signaling...
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 treatment study, increased expression of p44/p42 mitogen-activated protein kinase and HIF1α were independent predictors of response. For example, in a recent neoadjuvant treatment 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 and their associated kinase activity are constitutively expressed and activated throughout the tumor cell cycle. These cyclin E–associated cell cycle disruptions affect not only the G1 checkpoint but also those at S, G2, and M (13, 14). The overlap between points in the cell cycle in which cyclin E is deregulated and points in the cell cycle targeted by endocrine therapy raises the possibility that cyclin E modulation may be a predictor of response to endocrine therapy in breast cancer patients. Experimental models from several laboratories, including our own, have implicated the cyclin E–cyclin-dependent kinase 2 (CDK2) complex and their associated inhibitors, p21 and p27, as important mediators of antiestrogen therapy in cancer cells (10, 15). We have reported on resistance to fulvestrant mediated by the LMW forms of cyclin E, which are resistant to inhibition by p21 and p27 (10). Taken together, the data 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. 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 FACSCaliber 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

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### 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.

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.
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 Graph-Pad 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 Immobilon 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...
enrolled in an Institutional Review Board–approved proto-
col 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 anal-
ysis of cyclin E expression. Demographic, clinical, and path-
ologic data including the steroid-receptor status as well as
the low molecular cyclin E levels are described in Supple-
mental 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
yses 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. Differ-
ces were considered significant when the two-tailed Stu-
dent’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 cy-
lcin 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 out-
come 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 postmeno-
pausal 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 respon-
sible 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 con-
trols. Compared with control cells (CSSM), 25 nmol/L an-
drostenedione treatment resulted in a 3.9–± 1.3-fold
crease 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 par-
tially 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 in-
crease 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 pro-
gression 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 ef-
fective of the three inhibitors, we tested this drug in subse-
quent experiments. We investigated the effect of increasing
centrations of letrozole on the cyclin E–associated ki-
ase activity (Fig. 3A) and on the CDK2-associated kinase
activity (Supplementary Fig. S1). Androstenedione
ment 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 ki-
ase 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 ve-
icle-treated cells, whereas letrozole treatment blocked

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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 for p21 and p27. Although androstenedione treatment did not affect p21 binding to cyclin E, it slightly increased the binding to CDK2 by 1.5-fold, whereas letrozole treatment slightly decreased p21 binding to both cyclin E and CDK2. In contrast, although p27 protein levels remained unchanged after drug treatments, p27 binding to both cyclin E and CDK2 increased in a dose-dependent manner following letrozole treatment by up to 2-fold greater than the levels in androstenedione-treated cells. These results suggest that androstenedione-induced cell proliferation and G1 exit are mediated by an increase in phospho-CDK2 activity, and that letrozole inhibits these effects by preventing the androstenedione-induced increase in CDK2 activity and by inducing increased binding of p27 to cyclin E and CDK2 complexes.

LMW cyclin E, but not full-length cyclin E, overexpressing MCF-7/Ac1 cells partially override the letrozole inhibition of androstenedione-induced S-phase entry and androstenedione-induced CDK2 protein levels. Because overexpression of LMW cyclin E deregulates the G1-S transition, we
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 adenosviruses 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–overexpressing cells 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/Act1 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 (\( P = 0.0291 \), 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 treatment option for those postmenopausal breast cancer patients that may benefit from CDK inhibitors (i.e., overcoming 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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