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

Said Akił¹, Tuyen Bui¹, Hannah Wingate¹, Anna Biernacka¹, Stacy Moulder², Susan L. Tucker³, Kelly K. Hunt⁴, and Khandan Keyomarsi¹,⁵

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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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...
not only the G1 checkpoint but also those at S, G2, and M cycle. These cyclin E tutively expressed and activated throughout the tumor cell of cyclin E and their associated kinase activity are consti-

stability (9) increased cyclin E kinase activity, and increased genomic in-

targeted by endocrine therapy raises the possibility that cy-

mation for the design of more effective treatment strategies. For example, in a recent neoadjuvant treatment setting provides the opportunity to identify genes that could provide additional treatment options. The neoadju-

vant 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 HIF1a were independent predictors of protein kinase and HIF1a were independent predictors of resistance to letrozole (8). Taken together, these data sug-

ggest that identification and understanding of proteins that regulate response to AI treatment may provide critical infor-

nation 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 in-

stability (9–12). The low-molecular-weight (LMW) forms of cyclin E and their associated kinase activity are constitu-

tively 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 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 overexpres-

sion of LMW cyclin E leads to the lack of response to AI treatment in aromatase-overexpressing cells. We further ex-

amined the mechanism of this resistance and have pro-

posed targeted therapy to overcome it. Lastly, we provide evidence that breast cancer patients whose tumors over-

express LMW cyclin E are not responsive to AI treatment.

Materials and Methods

Chemicals. The AIs letrozole, exemestane, and anastro-

zole were provided by Astra Zeneca. These drugs were dis-

solved 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 mod-

ified Eagle’s medium with 5% fetal bovine serum, 1% pen-

icillin/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 resus-

pended 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 FACS-Caliber 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

Translational Relevance

Although letrozole treatment of postmenopausal es-

trogen 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 resis-

tance. Lastly, we show that breast cancer patients whose tumors overexpress LMW cyclin E are more like-

ly to recur after AI treatment compared with those with low or no expression of LMW cyclin E. These data sup-

port clinical investigation of cyclin-dependent kinase 2 inhibitor therapy for postmenopausal women with estrogen receptor–positive, LMW cyclin E–expressing breast cancer.

is also associated with resistance to endocrine therapy and suggests that inhibitors of signal transduction pathways could provide additional treatment options. The neoadju-

vant 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 HIF1a were independent predictors of resistance to letrozole (8). Taken together, these data sug-

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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 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 (19). This model system simulates 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 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

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Fig. 3. *In vitro* antiproliferative effect of increasing concentrations of letrozole in the presence of 25 nmol/L androstenedione (AD) on MCF-7/7Ac1 human breast cancer cells. A, letrozole blocks the androstenedione-induced increase in cyclin E–associated kinase activity in MCF-7/7Ac1 cells. MCF-7/7Ac1 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. 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.
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–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, the same samples were used to determine the block by roscovitine of androstenedione-induced CDK2 protein levels in uninfected and LacZ-infected cells, whereas letrozole completely blocks the 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 DMSO) or medium (CSSM) plus DMSO (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 the 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). 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 LMW forms of cyclin E. We show that this subgroup of patients has a poor prognosis, with a median survival time of only 3.25 years. We provide in vitro evidence that if these patients were to be treated with letrozole, it is likely that they will not respond effectively to this treatment.

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 CVC202).

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

Said Akli, Tuyen Bui, Hannah Wingate, et al.


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