

Targeting low molecular weight cyclin E (LMW-E) in breast cancer

Angela Nanos-Webb · Natalie A. Jabbour · Asha S. Multani · Hannah Wingate ·
Nassima Oumata · Hervé Galons · Benoît Joseph · Laurent Meijer ·
Kelly K. Hunt · Khandan Keyomarsi

Received: 5 January 2011 / Accepted: 10 June 2011 / Published online: 22 June 2011
© Springer Science+Business Media, LLC. 2011

Abstract Low molecular weight cyclin E (LMW-E) plays an important oncogenic role in breast cancer. LMW-E, which is not found in normal tissue, can promote the formation of aggressive tumors and can lead to increased genomic instability and tumorigenesis. Additionally, breast cancer patients whose tumors express LMW-E have a very poor prognosis. Therefore, we investigated LMW-E as a potential specific target for treatment either alone or in combination therapy. We hypothesized that because LMW-E binds to CDK2 more efficiently than full length cyclin E, resulting in increased activity, CDK inhibitors could be used to target tumors with LMW-E bound to CDK2. To test the hypothesis, an inducible full length and LMW-E MCF7-Tet-On system was established. Cyclin E (full length (EL) or LMW-E) is only expressed upon induction of the transgene. The doubling times of cells were

unchanged when the transgenes were induced. However, upon induction, the kinase activity associated with LMW-E was much higher than that in the EL induced cells or any of the uninduced cells. Additionally only the LMW-E induced cells underwent chromosome aberrations and increased polyploidy. By examining changes in proliferation and survival in cells with induced full length and LMW-E, CDK inhibitors alone were determined to be insufficient to specifically inhibit LMW-E expressing cells. However, in combination with Doxorubicin, the CDK inhibitor, Roscovitine (Seliciclib, CYC202), synergistically led to increased cell death in LMW-E expressing cells. Clinically, the combination of CDK inhibitors and chemotherapy such as Doxorubicin provides a viable personalized treatment strategy for those breast cancer patients whose tumors express the LMW-E.

Electronic supplementary material The online version of this article (doi:10.1007/s10549-011-1638-4) contains supplementary material, which is available to authorized users.

A. Nanos-Webb · N. A. Jabbour · K. Keyomarsi (✉)
Department of Experimental Radiation Oncology,
M. D. Anderson Cancer Center, 1515 Holcombe Boulevard,
Houston, TX 77030, USA
e-mail: kkeyomar@mdanderson.org

A. S. Multani
Department of Genetics, M. D. Anderson Cancer Center,
1515 Holcombe Boulevard, Houston, TX 77030, USA

H. Wingate · K. K. Hunt
Department of Surgical Oncology, M. D. Anderson Cancer
Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

N. Oumata
ManRos Therapeutics, Centre de Perharidy,
29680 Roscoff, France

Keywords Roscovitine · Seliciclib · CYC202 ·
Doxorubicin · LMW cyclin E · CDK inhibitors

H. Galons
Laboratoire de Chimie Organique 2, CNRS UMR8601, INSERM
U 648, Université Paris-Descartes, 4 avenue de l'Observatoire,
75270 Paris cedex 06, France

B. Joseph
Institut de Chimie et Biochimie Moléculaires et
Supramoléculaires, Université Claude Bernard-Lyon 1,
Bâtiment Curien, 43 Boulevard du 11 Novembre 1918,
69622 Villeurbanne, France

L. Meijer
CNRS, 'Protein Phosphorylation & Human Disease' Group,
Station Biologique, Bretagne, 29680 Roscoff, France

Introduction

In normal cells, cell division is a highly regulated process with several checks and balances; defects in this process can lead to genomic instability and ultimately cancer [1, 2]. In normal somatic cells, the cell cycle is driven by cyclin/CDK complexes and halted by genes that inhibit these complexes. The first major checkpoint occurs between the G1 and S phases, and the complex responsible for the G1 to S transition is the regulatory cyclin E bound to its catalytic binding partner, CDK2 [3–5]. In breast cancer, the gene for cyclin E has been shown to be amplified and overexpressed [6, 7]. Increased expression of cyclin E protein has been shown to drive cells into S phase more rapidly, thus increasing the rate of proliferation of these cells [8, 9]. Clinically, overexpression of cyclin E protein in breast cancer patients correlates with poor prognosis [10].

In tumor cells, full length cyclin E protein may be cleaved post-translationally, resulting in tumor-specific isoforms of cyclin E, known as low molecular weight cyclin E (LMW-E) [7, 11]. The cleavage of cyclin E into LMW-E is catalyzed by the serine protease, elastase [12]. LMW-E isoforms bind to CDK2 more efficiently than the full length form, and when LMW-E is in a complex with CDK2 it is resistant to the cyclin-dependent kinase inhibitors, p21^{cip1} and p27^{kip1} [13–15]. LMW-E isoforms are hyperactive in driving the cell cycle through an increase in CDK2 activity, thereby by-passing the G1/S checkpoint [12]. The more efficient LMW-Es are of great consequence, as their expression leads to not only greater CDK2 kinase activity but also increased genomic instability, and ultimately tumorigenesis [12, 14, 16].

Since LMW-E binds to CDK2 more efficiently than full length cyclin E, we hypothesized that LMW-E isoforms can be targeted specifically by CDK2 inhibitors either as single agents or in combination with chemotherapy, thereby causing differential cytotoxicity in tumor cells expressing LMW-E versus full length cyclin E. Several CDK inhibitors, such as Flavopiridol and Roscovitine (CYC202, Seliciclib), have been studied as single agents in clinical trials with relatively little success [17–21]. One of the reasons for poor clinical success of CDK inhibitors is that there are no screening strategies in place to identify those patients who are most likely to respond to these inhibitors. There is currently no clinical information on the use of LMW-E as a predictor of response to Seliciclib (or other CDK inhibitors). Since those breast cancer patients whose tumors overexpress LMW-E could benefit the most from these agents, there is need for pre-clinical studies examining the benefit of this agent alone and in combination with chemotherapy in cells that overexpress the LMW-Es as compared to cell lines that express only the full length cyclin E.

In this report we provide evidence that inducible expression of LMW-E, and not full length cyclin E, results in increased cyclin E-associated kinase activity and increased genomic instability, suggesting that the LMW-E expressing cells have a more transformed phenotype and should be used as a screening tool for identification of targeted therapy against LMW-E expressing tumors. Using this MCF7-Tet-On system with inducible full length and LMW-E, several CDK inhibitors were examined as single agents for improved efficacy against LMW-E expressing cells. The results of the study indicate that CDK inhibitors alone did not differentiate between induced and uninduced LMW-E expressers. However, when these inducible cells were sequentially treated with Roscovitine followed by Doxorubicin, a synergistic inhibition of survival of LMW-E expressing cells was discovered. Similar results were observed in breast cancer cells with endogenous overexpression of LMW-E. Therefore, CDK inhibition followed by Doxorubicin treatment provides a means to kill tumor cells expressing LMW-E. Clinically, breast cancer patients whose tumors express LMW-E would benefit the most from this treatment combination. Additionally, our data suggest that expression of LMW-E could provide a biomarker of response to targeted therapy directed at CDK2.

Methods

Preparation of MCF7-Tet-On inducible cells

MCF7-Tet-On cells were purchased from Clontech. Cyclin full length (EL) and low molecular weight (LMW) cyclin E constructs Truncation 1 (T1) and Truncation 2 (T2), described elsewhere [12], were subcloned into the inducible vector pTRE-Tight (Clontech). The MCF7-Tet-On cells were transfected with either EL, T1, or T2 using FuGENE transfection reagent (Invitrogen). Stable clones were selected using 100 µg/ml Hygromycin 48 h post-transfection and were maintained in complete media containing 50 µg/ml Hygromycin and 100 µg/ml G418. Positive clones were determined by western blot analysis in the presence or absence of 1 µg/ml Doxycycline using the cyclin E (HE-12; Santa Cruz Biotechnology) antibody (see western blot analysis).

Tet-tested fetal bovine serum was from Atlanta Biologicals, Inc., and HyQ MEM alpha-modification cell culture medium was from HyClone. Media was supplemented with 10 mM HEPES, non-essential amino acids, 2 mM L-glutamine, sodium pyruvate, hydrocortisone, and 10 µg/ml Cipro. MDA-MB-436 cells were obtained from ATCC and with culture conditions similar to MCF7 (regular fetal bovine serum was used instead of tet-tested fetal

bovine serum). All cells were cultured and treated at 37°C in a humidified incubator containing 6.5% CO₂–93.5% air.

Western blot analysis

Total cell lysates were prepared and analyzed by western blot analysis as previously described [22]. 35 µg of protein were run on SDS-PAGE. The samples were then transferred overnight at 35 mV at 4°C to Immobilon P membrane (Millipore). The membranes were blocked for 45–60 min at room temperature in blocking buffer (5% nonfat dried milk in TBST—20 mM Tris, 137 mM NaCl, 0.05% Tween 20, pH 7.6). After being washed in TBST, the blots were incubated in primary antibodies for 90–120 min, depending on the antibody optimization. Primary antibodies used were cyclin E (HE-12, Santa Cruz Biotechnology), Cdk2 (Transduction Labs), p21 (Ab-1, Calbiochem), p27 (K25020, BD Biosciences-Transduction Laboratories), p53 (Ab-6, Calbiochem), and actin (C4, Chemicon International). For the mouse primary antibodies, a secondary antibody from eBioscience (Mouse TrueBlot) was used. Blots were incubated with Mouse IgG TrueBlot at a 1:1750 dilution in blocking buffer for 45 min at room temperature, washed 6 × 10 min each for 1 h, and developed using the Renaissance chemiluminescence system as directed by the manufacturer (Perkin-Elmer Life Sciences, Inc.).

Immunofluorescence staining

MCF7 (induced or non-induced) cells were grown in six-well plates with cover slides and fixed in cold 4% neutral paraformaldehyde in PBS for 20 min at room temperature. The cells were then washed in PBS, permeabilized in cold methanol for 5 min, and blocked in 5% bovine serum albumin in PBS. Incubation with a primary antibody was carried out for 1 h at 37°C. Incubation with a secondary antibody was carried out for 1 h at room temperature, followed by staining of DNA with 4,6-diamidino-2-phenylindole (DAPI) for 10 min. Slides were mounted with Vectashield antifade medium (Vector Laboratories) after three washes with PBS and examined using a Leica DM4000B microscope equipped with ×63 Plan-Apochromat oil immersion objective. Selected images were cropped, aligned, and adjusted for contrast with Adobe Photoshop 5.0.

Histone H1 kinase analysis

For immunoprecipitation followed by an H1 kinase assay, 200 µg of whole cell extracts were used per immunoprecipitation with the polyclonal antibody to FLAG in lysis buffer containing 250 mM Tris buffer (pH 7.5), 1250 mM

NaCl, 0.05% NP40. The protein:antibody mixture was incubated with Sepharose protein A beads for 1 h, and the immunoprecipitates were washed four times with both lysis buffer and then kinase buffer [50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 0.1 mg/ml bovine serum albumin]. For the histone H1 kinase assay, the immunoprecipitates were then incubated with kinase assay buffer containing 60 µM cold ATP and 5 µCi of [³²P]ATP in a final volume of 50 µl for 30 min at 37°C. The products of the kinase reaction were analyzed on a 13% SDS-PAGE gel, which was then stained, destained, dried, and exposed to film. For quantitation, the protein bands corresponding to histone H1 were cut out, and radioactivity was measured using the scintillation counter.

Genomic instability analysis

MCF7-Tet-On cells transfected with inducible EL, T1, or T2 were cultured in the presence or absence of doxycycline (for induction) for 2 weeks before harvesting for chromosome preparation. Cytogenic metaphase spread analysis was performed following the standard procedures: cells were exposed to Colcemid (0.04 µg/ml) overnight due to the slow growth rate of the cells, subjected to hypotonic treatment (0.075 M KCl for 20–25 min at room temperature), and fixed in a mixture of methanol and acetic acid (3:1 by volume) [23]. Slides were stained in Giemsa stain, then examined blindly for chromosomal abnormalities. The slides were decoded after the scoring of aberrations was completed. From each sample, at least 30 metaphase spreads were analyzed. Representative spreads were captured using the Genetiscan imaging system.

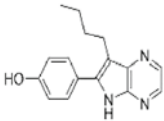
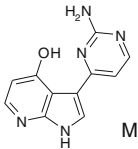
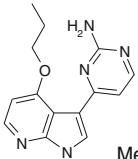
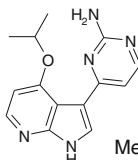
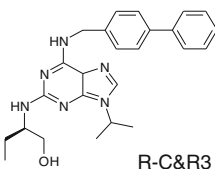
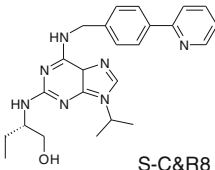
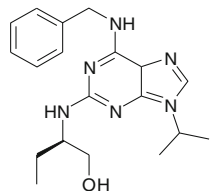
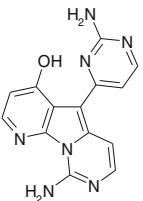
CDK inhibitors

All CDK inhibitors (see Table 1; Supplementary Tables 1 and 2 for drug names) used in this study were shipped to us from the laboratory of Dr. Laurent Meijer as either powder or DMSO solutions and to use for experiments they were further diluted in DMSO at a concentration of 10 mM.

MTT assay

In a 96-well, tissue culture-treated, flat-bottomed plate (Costar 3595), 5,000 cells/200 µl of cell suspension were plated on each well (Day 0). Twenty-four hours post-plating (Day 1), cells were treated with or without doxycycline (1 µg/ml) for induction of cyclin E. Forty-eight hours post-plating (Day 2), cells were treated with CDK inhibitors (see Table 1; Supplementary Tables 1 and 2 for drug names) at varying concentrations with or without doxycycline (1 µg/ml). The cells were incubated with CDK inhibitors plus doxycycline at 37°C for 3 days (until Day

Table 1 IC₅₀ values of structural classes of CDK2 inhibitors analyzed for proliferation and survival

| Class | Compound | Tet | MTT assay | | | HTCA | | |
|------------------|---|-----|--------------|--------------|--------------|---------------|--------------|--------------|
| | | | EL | T1 | T2 | EL | T1 | T2 |
| Pyrrolo-pyrazine |  | OFF | 5 μM | 5 μM | 5 μM | 18.97 μM ± nd | nd | 9.23 μM ± nd |
| | | ON | 5 μM | 5 μM | 5 μM | 21.54 μM ± nd | nd | 9.23 μM ± nd |
| Aloisine A | | | | | | | | |
| Meriolins |  | OFF | 0.54 μM | nd | 0.46 μM ± nd | 0.44 μM 0.14 | 0.26 μM 0.02 | 0.37 μM 0.08 |
| | | ON | 0.53 μM | nd | 0.63 μM 0.05 | 0.42 μM 0.17 | 0.26 μM 0.02 | 0.40 μM 0.06 |
| |  | OFF | 0.32 μM ± nd | 0.14 μM ± nd | <1 μM | 25 nM ± nd | 25 nM ± nd | 44 nM ± nd |
| | | ON | 0.32 μM ± nd | 0.15 μM ± nd | <1 μM | 25 nM ± nd | 25 nM ± nd | 37 nM ± nd |
| |  | OFF | 100 nM ± nd | 50 nM ± nd | <1 μM | 4.8 nM ± nd | 7.2 nM ± nd | 24 nM ± nd |
| | | ON | 100 nM ± nd | 50 nM ± nd | <1 μM | 3.6 nM ± nd | 7.2 nM ± nd | 27 nM ± nd |
| Purines |  | OFF | 1.75 μM 1.06 | nd | 1.44 μM ± nd | 0.80 μM ± nd | nd | 0.65 μM ± nd |
| | | ON | 1.75 μM 1.06 | nd | 2.10 μM ± nd | 0.80 μM ± nd | nd | 0.65 μM ± nd |
| |  | OFF | 0.62 μM ± nd | 1.20 μM ± nd | 1 μM ± nd | 0.13 μM ± nd | 0.53 μM ± nd | 0.27 μM ± nd |
| | | ON | 0.57 μM ± nd | 1.14 μM ± nd | 1 μM ± nd | 0.15 μM ± nd | 0.53 μM ± nd | 0.29 μM ± nd |
| |  | OFF | 14.4 μM 15.3 | 10.95 μM 8.9 | 11.2 μM 6.5 | 10.1 μM 9.2 | 5.4 μM 1.04 | 7.4 μM 0.3 |
| | | ON | 16.1 μM 13.9 | 15.2 μM 14.2 | 12.5 μM 7.5 | 8.9 μM 3.8 | 5.9 μM 0.99 | 7.9 μM 1.0 |
| Variolin B |  | OFF | 880 nM ± nd | 390 nM ± nd | <1 μM | 60 nM ± nd | 80 nM ± nd | 380 nM ± nd |
| | | ON | 500 nM ± nd | 390 nM ± nd | <1 μM | 33 nM ± nd | 110 nM ± nd | 320 nM ± nd |

IC₅₀ values (± the standard deviation) were determined using the MTT proliferation assay and the HTCA survival assay in the presence or absence of cyclin EL, T1, and T2 induction. CDK2 inhibitor structures have been categorized into four classes, Pyrrolo-pyrazine, Meriolins, Purines, and Variolin B. “OFF” indicates non-induced, while “ON” indicates the induction of the transgene (full length cyclin E-EL, or LMW-E-T1 or T2). If the > sign is used, not enough drug was present to determine the IC₅₀ value. nd = not determined. No significant difference was seen between the induced and non-induced cyclin E samples. However, the cells responded differently to the various classes of CDK2 inhibitors

5). At day 5, the cells were immediately harvested. Harvesting was performed by addition of 50 μ l per well of 2.5 mg/ml MTT (Sigma M-5655—methylthiazolyldiphenyl-tetrazolium bromide) in serum-free media. The plates were sealed in foil and incubated at 37°C for 4 h and media removed. 100 μ l solubilization solution (0.04 N HCl, 1% SDS, in isopropyl alcohol) was added to each well. Plates were re-sealed in foil and lightly rocked at room temperature for 2–3 h. After solubilization, plates were read at 590 nm by the Perkin-Elmer–Wallac–Victor3 1420 multi-label counter.

High throughput clonogenic assay (HTCA)

In a 96-well, tissue culture-treated, flat-bottomed plate (Costar 3595), 500 cells/200 μ l of cell suspension were added to each well (Day 0). Twenty-four hours post-plating (Day 1), cells were treated with or without doxycycline (1 μ g/ml) for induction of cyclin E. Forty-eight hours post-plating (Day 2), cells were treated with the indicated drugs (i.e., Table 1) at varying concentrations in the presence or absence of doxycycline (1 μ g/ml) for an additional 24 h (until Day 3). On day 3, the drugs were removed and each well was rinsed with sterile PBS (three times), and fresh drug-free complete alpha media (but still with or without doxycycline) was added. This media was changed every 48 h for 7–10 days (depending on the confluency of the control, non-drug exposed cells). When the control, non-drug treated cells have achieved near full confluency, the cells are harvested as per the MTT assay.

Combination treatments with Roscovitine/Doxorubicin

MCF7-Tet-On EL and T1 cells were plated at 5,000 cells per well on 96-well plates. Twenty-four hours following plating, the expression of the EL and T1 transgene were induced by 1 μ g/ml of doxycycline. Induced cells were kept in doxycycline for the duration of the experiment. Non-induced cells were cultured in doxycycline-free media. After 24 h of induction the cells were treated sequentially with Drug A for 24 h and then Drug B for 48 h. After 48 h of Drug B cells were cultured in drug-free media for the duration of the experiment. Both Doxorubicin and Roscovitine were used as Drug A and Drug B. The specific concentrations for Doxorubicin and Roscovitine indicated for each cell line are equatoxic concentrations based on the inhibitory concentrations measured from single drug treatment for each cell line (data not shown). When Doxorubicin was Drug A, MCF7-EL cells were treated at concentrations of 5.2, 8.8, and 20 nM, and MCF7-T1 cells were treated at concentrations of 5, 16, and 30 nM. When Roscovitine was Drug A, both MCF7-EL and MCF7-T1 cells were treated at concentrations of 10,

12.5, and 22 μ M. When Doxorubicin was Drug B, MCF7-EL cells were treated at a concentration range of 1–30 nM, while MCF7-T1 cells treated at a concentration range of 1–25 nM. When Roscovitine was Drug B, both MCF7-EL and MCF-T1 cells were treated at a concentration range of 1–20 μ M. Both induced and non-induced cells were treated with the same concentrations for each cell line. Twelve days after plating, an MTT assay was performed and the plates were read at 590 nm. Synergy was calculated using the computer program *CalcuSyn*.

Results

Induction of LMW-E, but not full length cyclin E, results in higher associated kinase activity

In previous studies aimed at examining the oncogenic role of LMW-E, we generated normal and tumor cell lines that would stably overexpress either full length cyclin E or LMW-Es [14, 15]. While these stable cell lines were instrumental in our initial studies, they are not suitable for pharmacological analysis due to clonal variability (data not shown). In order to interrogate the role of LMW-Es in mediating altered response to CDK inhibitors, we generated an inducible model system where the expression of full length and LMW-Es could be manipulated (off or on) using the same cell line. To this end, we used the MCF7-Tet-On system to inducibly express EL (full length cyclin E), T1, and T2 (LMW-E truncations 1 and 2, respectively). MCF7 cells were chosen because these cells lack endogenous LMW cyclin E.

Once the inducible cells were generated, we examined their inducibility as a function of dose (Fig. 1a) and time (Fig. 1b) of doxycycline treatment. Western blot analysis with cyclin E revealed that upon induction with doxycycline that all three transgenes (EL, T1, and T2) were induced to maximal levels at 1.0 μ g/ml (Fig. 1a) and upon 24 h of treatment (Fig. 1b). Additionally, no changes in expression of CDK2, p21 or p27 were noted when the transgenes were induced (Fig. 1a). Upon induction of EL, the cells also generated LMW-Es (T1 and T2) (Fig. 1a and b, EL panels). The reason for expression of LMW-E upon induction of EL is due to induction of endogenous elastase levels. Our results revealed that all cyclin E isoforms when overexpressed result in increase in elastase levels (see Supplemental Figure 1) thereby resulting in an increase in the LMW-E to cyclin EL ratio. We see these forms appear when induction of the cyclin E transgene occurs over time, suggesting that there is a positive feedback loop between EL and elastase in these cells, such that when EL is induced, elastase levels are elevated resulting in cleavage of EL into LMW-E (Supplemental Figure 1).

We next assessed if induction of EL, T1 or T2 would result in changes in the rate of proliferation of cells. For these analyses, cells were plated at low density in the presence or absence of doxycycline and enumerated every other day for 14 days. The results revealed that induction of the transgene did not cause a significant change in the rate of proliferation of cells (Fig. 1c). We also examined the localization of the transgene and showed that while induction of EL and T1 resulted in predominantly nuclear accumulation of cyclin E, that when T2 is induced, it is localized to both the nuclear and cytoplasmic compartments (Fig. 1d). The cause of cytoplasmic localization of LMW-E is due to the truncation of the nuclear localization sequences, which are present in full length cyclin E [24].

Next, we measured the level of cyclin E-associated kinase activity in all three inducible cell lines and found that while induction of cyclin EL shows relatively no change in cyclin E-associated kinase activity that induction of both T1 and T2 led to 3–5-fold higher kinase activity when compared to EL or non-induced cells (Fig. 1e). These results suggest that induction of LMW-E by itself, in the absence of concomitant increases in CDK2 levels or downregulation of p21 and p27, is sufficient to increase the activity of the transgene as compared to full length cyclin E.

Induction of LMW but not full length cyclin E contributes to genomic instability

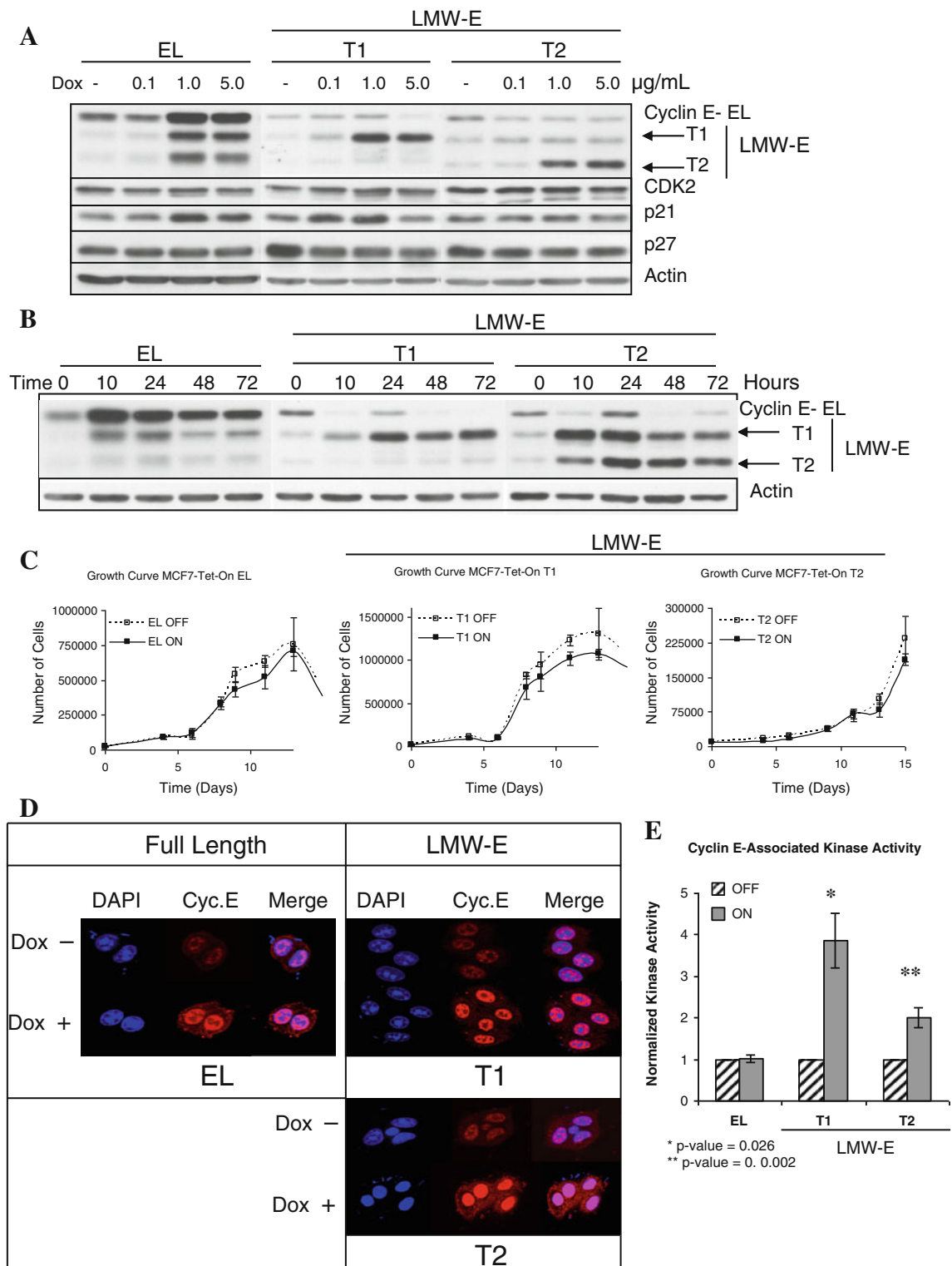
One of the hallmarks of cancer is genomic instability and we have shown that LMW-E expression correlates with the presence of polyploidy in breast cancer patient samples [14]. Here we asked if induction of full length cyclin E or LMW-E could result in genomic instability. For these studies, we cultured EL and LMW-E (T1 and T2) cells in the presence and absence of doxycycline for 14 consecutive days and subjected the cells to cytogenetic analysis of the metaphase chromosomes. This analysis revealed that LMW, but not full length, cyclin E resulted in a significant increase in the number of chromosome aberrations per metaphase spread (Fig. 2a, b) which were not observed in the EL induced cells or any of the non-induced cells. The aberrations in these chromosomes were due to increases in chromosomal breaks, fusions, fragments, dicentric chromosomes, and polyploidy in LMW (T1 and T2) cyclin E, but not the full length form (EL) (Fig. 2a). LMW-E also increased the polyploidy of these cells (Fig. 2b). These results clearly show that overexpression of the LMW-E (both T1 and T2) is sufficient to induce genomic instability. It should be noted that while these cells were all cultured (induced versus non-induced) for 2 weeks (4 passages all on the same days) before being subjected to genomic instability analysis, initial pilot experiments were

Fig. 1 LMW-E has an increased level of activity independent of the growth rate. **a** MCF7-Tet-On Cyclin EL, T1, and T2 cells were induced with increasing doxycycline concentrations at 24 h. The expression of cyclin EL, T1, and T2 increased with increasing doxycycline. Cyclin E, p21, p27, and CDK2 protein levels were examined by Western blot analysis. Cyclin E protein levels were examined by western blot analysis using cyclin E HE-12 antibody, which detects both full length and LMW cyclin E. Actin was used as a loading control. **b** MCF7-Tet-On Cyclin EL, T1, and T2 were induced with 1 $\mu\text{g/ml}$ doxycycline over 0, 10, 24, 48, and 72 h. The expression of cyclin EL, T1, and T2 remained steady over this time frame. Actin was used as a loading control. **c** Growth curves were performed in triplicate by plating 25,000 cells per plate initially and counting every 2 days. “ON” cells were exposed to 1 $\mu\text{g/ml}$ doxycycline during the duration of the experiment. No significant difference in growth was seen between cyclin E “OFF” and “ON” cells. **d** Immunofluorescence was performed in the presence or absence of doxycycline. Cyclin E expression in doxycycline (–) cells is likely due to the presence of endogenous cyclin E. **e** A Histone H1-kinase assay was performed using FLAG to immunoprecipitate the exogenous cyclin E followed by a kinase assay using histone H1 as the substrate. Histone H1 is phosphorylated by cyclin E/CDK2 allowing cyclin E-associated kinase activity to be measured quantitatively

performed with only 24 h of induction, and results (not shown) were comparable, indicating that these genomic aberrations do not accumulate over passage time. Additionally, since induction LMW-E results in a higher cyclin E-associated kinase activity as compared to full length cyclin E, it provides rationale for using this model system in drug screening assays targeting the activity of the cyclin E-associated kinase.

Inhibition of proliferation and viability by different classes of CDK2 inhibitors

Roscovitine, a well established small molecule CDK2 inhibitor which competes with ATP for CDK2's binding site, is currently in Phase II clinical trials [25–28]. Initially, we set out to examine if Roscovitine could differentially inhibit the LMW cyclin E/CDK2 complexes versus full length cyclin E/CDK2 complexes. To this end, cell extracts from insect cells co-infected with CDK2 and each of the three cyclin E forms (EL, T1, and T2) were subjected to in vitro kinase assays with histone H1 as a substrate, with 15 μM ATP and in the presence of the increasing concentrations of Roscovitine. The results (Supplementary Figure 2) clearly show that Roscovitine had a higher efficacy toward the LMW than the EL cyclin E/CDK2 complexes with IC_{50}s being 2–3-fold lower for the LMW-E containing complexes. This initial study propelled us to examine other CDK2 inhibitors in cultured cells and examine their growth inhibitory potential toward cells overexpressing either EL or the LMW-E forms. Since its development, several different analogues of Roscovitine as well as structurally unrelated CDK inhibitors have been identified [21]. These inhibitors include for example



purines, meriolines [29, 30], variolin, and pyrido-pyrazines [31]. We set out to examine the cytostatic and cytotoxic potential of these classes of agents in our panel of inducible full length and LMW-E MCF7 cell lines using MTT assay (to examine growth inhibition) and high throughput

clonogenic assay (HTCA, to examine cytotoxicity). We hypothesized that LMW-E expression would provide a useful biomarker in determining sensitivity to CDK2 inhibition. To test this, we used both MTT and HTCA to screen several representative CDK2 inhibitors. Our hope

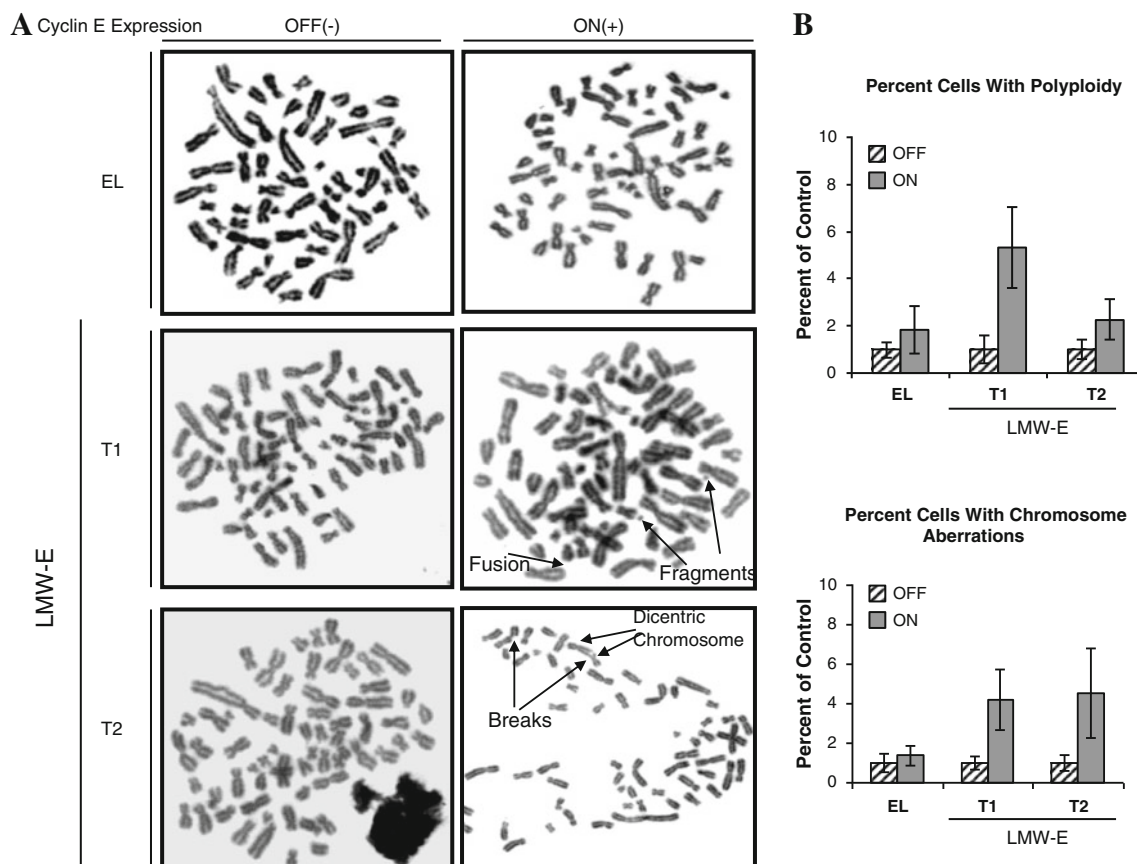


Fig. 2 Induction of LMW but not full length cyclin E contributes to genomic instability. **a** Metaphase spreads were used to examine changes between cyclin E induced (ON) and non-induced (OFF) in genomic instability. **b** *Panel one* Quantitative analysis of the percent

of cells with polyploidy was performed from metaphase spread data. *Panel two* Quantitative analysis of the percent of cells with chromosome aberrations was performed. EL-Full length, T1-LMW-E truncation 1, T2-LMW-E truncation 2

was to find a drug that mediated cytotoxicity specifically in the MCF7-Tet-On cells with induced T1 and T2 (LMW cyclin E) but not the EL induced or the non-induced controls, as we had observed in our in vitro kinase assays (Supplementary Figure 2). For these experiments, EL and LMW-E cell lines under induced and non-induced conditions were treated with 41 different small molecule inhibitors (Table 1; Supplemental Tables 1 and 2) and subjected to short term MTT or long term HTCA assays to measure growth inhibition and cytotoxicity, respectively. The structures and IC_{50} values of 8 representative inhibitors are depicted in Table 1 and dose-response curves for each agent in each cell line are shown in Fig. 3. Supplemental Tables 1 and 2 depict the IC_{50} values of the additional 33 inhibitors that were examined. These results show that the most potent class of kinase inhibitors are Meriolins with growth inhibitory IC_{50} values (in EL cells) ranging from 100 nM to 0.54 μ M and cytotoxic IC_{50} s ranging from 3.6 nM to 0.44 μ M. The marine sponge-derived Variolin B is the next potent kinase inhibitor with growth inhibitory IC_{50} at about 1 μ M and cytotoxic IC_{50} s at 50 nM.

Roscovitin was one of the least potent CDK2 inhibitors with growth inhibitory IC_{50} at 15 μ M and cytotoxic IC_{50} at 10 μ M. These results suggest that the Meriolins provide a very potent class of kinase inhibitors with profound growth inhibition and cytotoxic profiles. Another key finding from our studies was that induction of EL or the LMW-E was not sufficient to mediate a targeted/specific change in proliferation or survival between cells that overexpressed cyclin E (either full length or LMW-E) as compared to cells with no overexpression of cyclin E. However, since the LMW-E induction leads to genomic instability (Fig. 2), we asked if combination treatment with CDK2 inhibitors and a DNA damaging agent would be more effective than CDK2-inhibitors alone when LMW-E was induced.

CDK inhibition along with chemotherapy can lead to synergistic inhibition of LMW-E expressing cells

We next hypothesized that combination of a CDK inhibitor and a chemotherapeutic agent would have synergistic cytotoxicity when LMW-E is induced as compared to

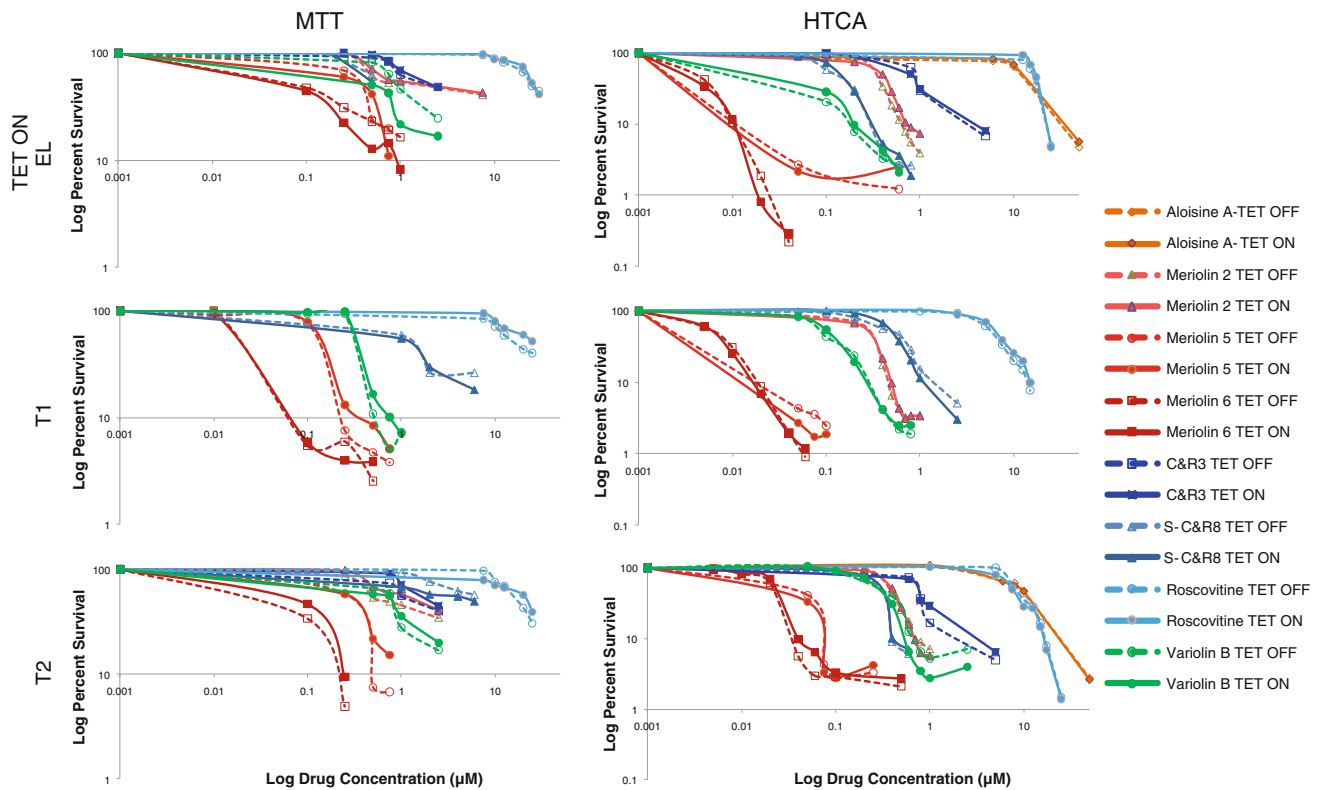


Fig. 3 Comparative analysis of CDK2-inhibitors on growth and survival. MCF7-Tet-On EL, T1, or T2 cells were treated with several classes of CDK inhibitors (see Table 1 for classifications and IC_{50} values) in the presence or absence of doxycycline to control expression of the transgene. Pyrrolo-pyrazine (*orange*), Meriolins

(*red*), Purines (*blue*), and Variolin B (*green*) were tested using the MTT proliferation assay and HTCA survival assay. Regardless of cyclin E induction, Meriolins exhibit the lowest IC_{50} values. The HTCA assay required lower drug concentrations than the MTT assay

non-induced or EL induced cells. To test this hypothesis, we used Doxorubicin as the DNA damaging agent, as it is one of the drugs currently in the clinic for the treatment of breast cancer. For this analysis, we treated EL and LMW-E (T1) inducible cells sequentially with Roscovitine first (24 h) followed by Doxorubicin (48 h) (R → D) or Doxorubicin followed by Roscovitine treatment (D → R). HTCA was used to measure the cell survival with drug combinations in the presence or absence of cyclin E induction. Synergy between the combinations was assessed using Calcsyn software. The results revealed that Roscovitine followed by Doxorubicin showed synergy with LMW cyclin E-T1 induction, but showed additivity without induction (Fig. 4a). However, this combination did not show synergy upon induction of full length cyclin EL, an indication that Roscovitine followed by Doxorubicin could be a useful treatment strategy among patients whose tumors express LMW cyclin E. Doxorubicin followed by Roscovitine did not have the same effect as the reverse combination. In fact, Doxorubicin followed by Roscovitine seemed to be antagonistic (Fig. 4a) in both cell lines under induced or non-induced conditions. Specifically, the

fraction of cell death in the Roscovitine followed by Doxorubicin was significantly higher in the induced LMW cyclin E-T1 samples than the non-induced ($P < 0.001$) or the induced full length cyclin E controls ($P = 0.13$) (Fig. 4b).

We next examined the sequential combination of Roscovitine with Doxorubicin in another breast cancer cell line with endogenous overexpression of LMW-E (Fig. 5). We therefore investigated the effects of combining Roscovitine with Doxorubicin in the MDA-MB-436 cell line, which is a triple negative cell line as it lacks expression of estrogen and progesterone receptors and the HER-2 oncogene. HTCA assays were used to compare cytotoxic effects of each drug alone or the combination of Roscovitine with Doxorubicin. When given individually, Roscovitine and Doxorubicin showed a dose-dependent reduction in cell viability in this cell line (data not shown). However, with the sequential combination of Roscovitine followed by Doxorubicin, there was significantly decreased cell viability compared with either agent alone only in the MDA-MB-436 cell line. Additionally, the sequence of addition of the agents is critical. We have found that Roscovitine

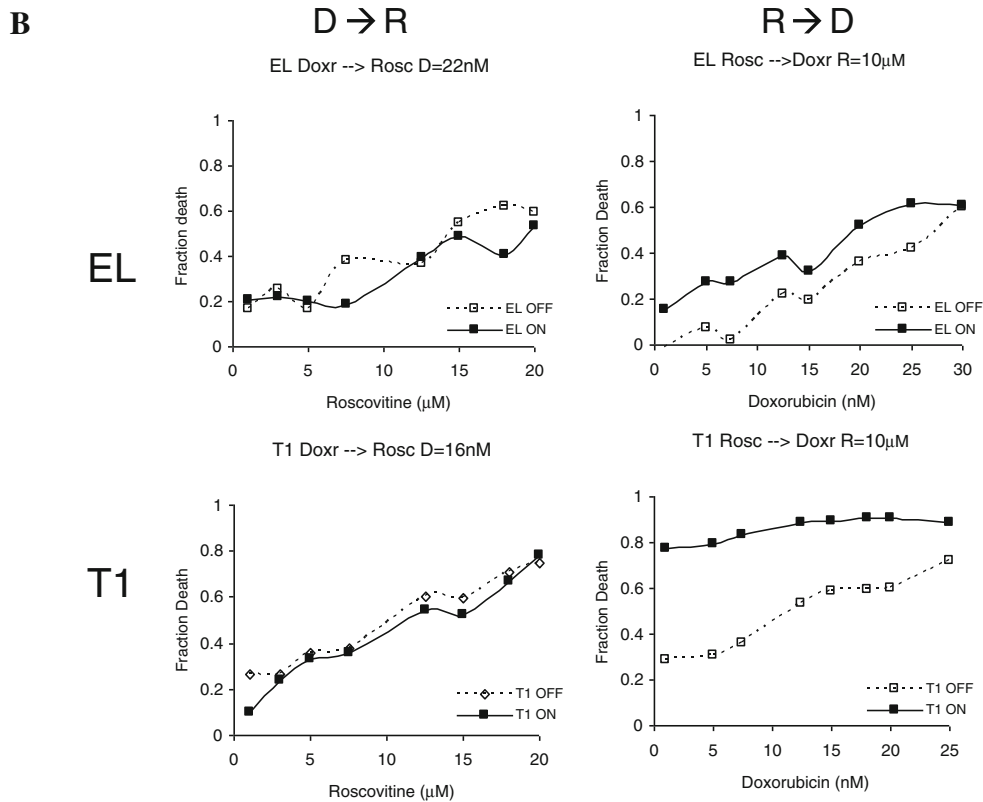
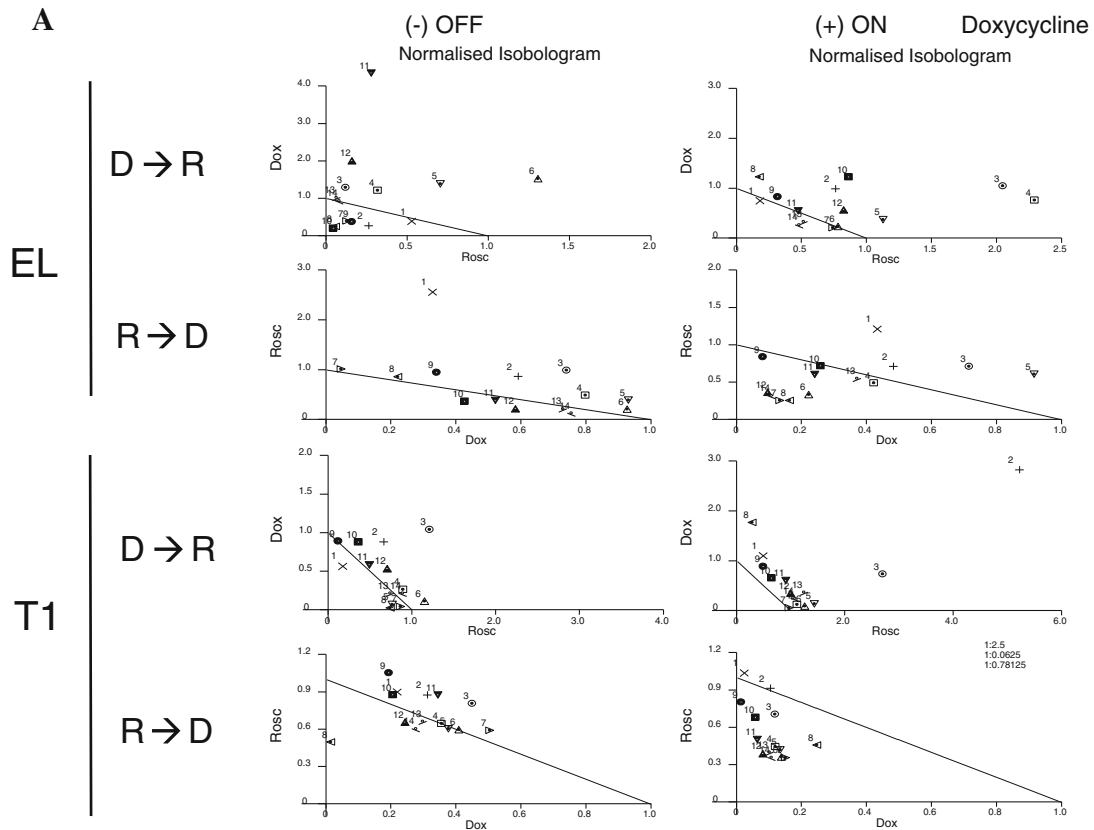


Fig. 4 Combination treatment of Roscovitine followed by Doxorubicin induces synergistic cell death in MCF7 cells. MCF7-Tet-On EL and T1 cells were exposed to either Doxorubicin followed by Roscovitine (D → R) or Roscovitine followed by Doxorubicin (R → D) and cell death was measured using the HTCA survival assay. **a** Drug combinations were measured for additivity, synergism, or antagonism using an isobologram analyzed by CalcuSyn software. R → D in the T1-induced cells appeared to be the only synergistic combination of all combinations and cell lines tested. **b** The fraction of cell death was measured using the method described, only T1-induced cells treated with Roscovitine followed by Doxorubicin exhibit increase cell death over their non-induced counterpart

should be administered first, followed by the chemotherapeutic agent (Doxorubicin) for the combination to show synergistic cytotoxicity (Fig. 5a). We also analyzed the data according to the combination index (defined through isobolograms), which also shows synergistic cytotoxicity between the two agents (Fig. 5b). If the combination index (CI) falls on the horizontal red line, the effect of the combination is additive; if the CI is above the line, the effect is antagonistic, and if the CI is below the line, then the effect is synergistic. The results clearly show that sequential

combination of Roscovitine with Doxorubicin is synergistic (i.e., CI is below the red line) while that of Doxorubicin followed by Roscovitine is antagonistic (i.e., CI above the red line). Western blot analysis with cyclin E antibody clearly shows that this cell line endogenously expresses the full complement of the LMW-E forms as compared to a normal cell line (Fig. 5c). Collectively, the results in Figs. 4 and 5 suggest that pretreatment with Roscovitine followed by Doxorubicin treatment could provide a targeted treatment option for LMW cyclin E expressing breast cancer.

Discussion

The LMW-E isoforms have been implicated in metastatic mammary cancer in transgenic animals, and their expression is also correlated with poor prognosis in breast cancer patients [10, 16]. In this study, we set out to examine if LMW-E could be specifically targeted as compared to EL expression. To this end, we generated inducible MCF7 breast cancer cell lines expressing either the full length or

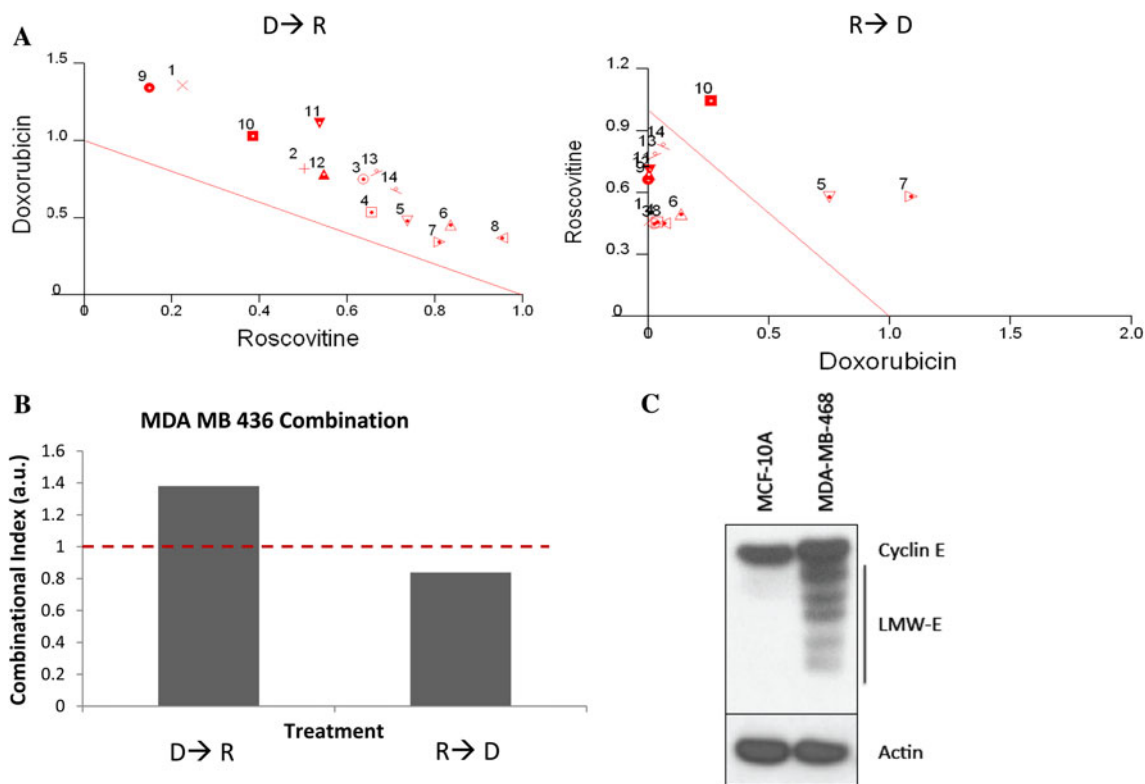


Fig. 5 Combination treatment of Roscovitine followed by Doxorubicin induces synergistic cell death in the LMW-E expressing cell line MDA-MB-436. MDA-MB-436 cells were exposed to either Doxorubicin followed by Roscovitine (D → R) or Roscovitine followed by Doxorubicin (R → D) and cell death was measured using the HTCA survival assay. **a** Drug combinations were measured for additivity, synergism, or antagonism using an isobologram analyzed by CalcuSyn software. **b** The combination index was calculated for all

different drug concentrations and averaged by CalcuSyn software which shows that only the R → D in MDA-MB-436 cells is synergistic with the combination index values being lower than 1. **c** Western blot analysis of lysates from MDA-MB-436 (tumor) and MCF-10A (normal-immortalized) with cyclin E depicts that MDA-MB-436 expresses the full range of all the LMW-E forms as well as EL while the normal cells only express EL

each of the LMW-E forms under the control of doxycycline. We show that upon induction of LMW-E, but not EL, that the cyclin E-associated kinase activity is induced and the cells undergo genomic instability. Additionally, the different LMW-E forms deregulate cells differently; specifically overexpression of T1 isoform causes deregulation of S phase, while overexpression of the T2 isoforms causes deregulation of the G2/M phase [32]. As such, this inducible cell line provides an ideal model system to examine the specificity of CDK2 inhibitors for LMW-E versus full length cyclin E. We examined a total of 41 different CDK2 inhibitors (different in terms of scaffold structure, selectivity, and potency as kinase inhibitors) in each cell line, under induced and non-induced conditions. Our results revealed that the induction of either full length or LMW-E cyclin E was not sufficient to mediate a difference in growth inhibition or cytotoxic potential of these CDK2 inhibitors as single agents. However, since induction of LMW-E induces genomic instability, we hypothesized that combinatory treatment with a CDK2 inhibitor and a DNA damaging agent could be synergistic only in cells that express the LMW-E forms, and not full length cyclin E. Our results revealed that sequential treatment of cells with Roscovitine followed by Doxorubicin (and not the other way around) was synergistic only in the LMW-E induced cells and not in any of the non-induced or EL cells. Similar results were obtained when the combination of Roscovitine followed by Doxorubicin was administered in a triple negative breast cancer cell line which endogenously over-expresses the full complement of the LMW-E forms. Collectively, these results suggest that CDK2 might be a viable target, only if the patient population is selected based on the expression of LMW-E in their tumors, and if a CDK2 inhibitor is administered in a combination treatment.

LMW-E binds to CDK2 more efficiently than the full length form of cyclin E, resulting in increased CDK2 activity [13, 15]. Based on this knowledge, we believe a proficient method for targeting LMW-E is through CDK inhibition. The concept of CDK inhibition goes back to 1999, when Chen et al. [33] used short peptide motifs that inhibited the ability of CDK2 to phosphorylate its substrates in transformed cells resulting in cell death. Since then, small molecule inhibitors relatively specific to CDKs have been designed and are in clinical trials [17–21]. In our investigation, CDK2 inhibitors as single agents did not seem to target LMW-E expressing cells over their counterparts. This, along with CDK inhibitors' relative lack of success as single agents in clinical trials, led us to do combination studies with CDK inhibitors and other chemotherapies.

Given the recent finding that cells with genomic instability are not as prone to survive and multiply [34], we set out to utilize the ability of LMW-E to induce genomic

instability to sensitize these cells to other agents, resulting in increased cell death. Roscovitine, through inhibition of CDK2, has been shown to lead to anaphase catastrophe and result in increased cell death [35]. Roscovitine and Doxorubicin individually have known dose-limiting toxicities. We examined different sequential combinations of these drugs to determine if synergism can be achieved. Treatment with Roscovitine, followed by Doxorubicin resulted in synergism in cyclin E overexpressing cells, in full length and to a greater extent in LMW-E expressing cells (Figs. 4, 5). The reason that LMW-E overexpressing breast cancer cells are more sensitive to sequential treatment of Roscovitine followed by Doxorubicin is that Roscovitine treatment arrests these cells in the G2 phase of the cell cycle, where they will be most responsive to the toxic effects of chemotherapy. We have already provided proof of concept of this mechanism with a sarcoma cell line [36]. In that study, we showed that treatment with Roscovitine and Doxorubicin induces a profound G2 arrest only in sarcoma cell lines and not in MCF-10A cells. The results show that Doxorubicin either alone or in combination with Roscovitine results in only 30–50% cytotoxicity in normal cells. However, the same treatment in the sarcoma cells results in over 95% cytotoxicity. The synergistic action of the combination treatment in the sarcoma cell line is clearly apparent as either drug alone did not result in any appreciable toxicity. We also showed that, while the normal cells were mainly arrested in G1, sarcoma cells accumulated in G2/M following the treatment. These results raise the hypothesis that the mechanism that mediates the synergistic cytotoxicity of Roscovitine followed by Doxorubicin is likely to be through alteration in the G2/M transition. We propose here that treating breast cancer cells, which have high LMW-E, sequentially with Roscovitine followed by chemotherapy, takes advantage of compromised mitotic exit by first arresting cells, which already harbor a damaged genome, in G2/M and then attacking those cells with DNA damaging compounds. This combination treatment causes “mitotic catastrophe” mediated cell death.

The clinical experience with Roscovitine (i.e., Seliciclib) as a single agent has not been very promising [17, 26–28]. To date a total of 321 cancer patients, with different malignancies, have been treated with Seliciclib to investigate the tolerability of increasing doses of the drug and establishment of maximal tolerable dose and response. The anti-tumor activity of Seliciclib as a single agent has been monitored in 77 patients with hepatocellular, non-small cell lung cancer (NSCLC), thymoma, adenocarcinoma UP, adrenal cancer, papillary cystadenoma of ovary, and parotid cylindroma. Of these 77 patients, one partial response was seen in hepatocellular carcinoma, 2 prolonged stable disease observed in NSCLC (14 and >18 months), while stable disease was seen in the remaining solid tumors.

Currently, Seliciclib is being developed clinically to be used in multiple treatment modalities with NSCLC and nasopharyngeal cancer. It should be noted that none of the treated patients (in either phase I or II) were pre-selected based on altered expression of cyclin E or CDK2. The data presented in this manuscript provides rationale for preselecting patients for cyclin E and CDK2 expression for treatment with CDK2 inhibitors in sequential and/or combination with chemotherapeutics. Because LMW-E expressing tumor cells exhibit the highest level of synergy between Roscovitine followed by Doxorubicin treatment, patients exhibiting LMW-E expressing cancers would perhaps benefit most from a pretreatment with Roscovitine followed by treatment with Doxorubicin.

Acknowledgments We would like to thank Tuyen Bui for his assistance on the generation of the MCF7-Tet-On T2 cell line. Also, we would like to thank Yanna Liu for the immunofluorescence of the MCF7-Tet-On cells. We would also like to thank Jin Ma for the time spent preparing the MCF7-Tet-On samples for metaphase spread analysis. This research was supported by NIH grants CA87458, P50CA116199, Susan G. Komen grants KG100521 and KG100876 to Khandan Keyomarsi and Kelly K. Hunt, by the CCTS T32 grant to Natalie Jabbour through the NIH Clinical and Translational Award TL1 RR024147, by NCI CCSG grant CA16672 to M.D. Anderson Cancer Center, “Cancéropole Grand-Ouest”, the “Association pour la Recherche sur le Cancer” (ARC-1092), the “Ligue Nationale contre le Cancer (Comité Grand-Ouest)” to Laurent Meijer.

References

- Hartwell LH, Kastan MB (1994) Cell cycle control and cancer. *Science* 266:1821–1828
- Myung K, Datta A, Kolodner RD (2001) Suppression of spontaneous chromosomal rearrangements by S phase checkpoint functions in *Saccharomyces cerevisiae*. *Cell* 104:397–408
- Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M (1995) Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol* 15:2612–2624
- Koff A, Cross F, Fisher A, Schumacher J, Leguellec K, Philippe M, Roberts JM (1991) Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. *Cell* 66:1217–12285
- Koff A, Giordano A, Desai D, Yamashita K, Harper JW, Elledge S, Nishimoto T, Morgan DO, Franza BR, Roberts JM (1992) Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science* 257:1689–1694
- Keyomarsi K, Pardee AB (1993) Redundant cyclin overexpression and gene amplification in breast cancer cells. *Proc Natl Acad Sci USA* 90:1112–1116
- Keyomarsi K, O’Leary N, Molnar G, Lees E, Fingert HJ, Pardee AB (1994) Cyclin E, a potential prognostic marker for breast cancer. *Cancer Res* 54:380–3858
- Resnitzky D, Gossen M, Bujard H, Reed SI (1994) Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol Cell Biol* 14:1669–1679
- Sauer K, Lehner CF (1995) The role of cyclin E in the regulation of entry into S phase. *Prog Cell Cycle Res* 1:125–13910
- Keyomarsi K, Tucker SL, Buchholz TA, Callister M, Ding Y, Hortobagyi GN, Bedrosian I, Knickerbocker C, Toyofuku W, Lowe M, Herliczek TW, Bacus SS (2002) Cyclin E and survival in patients with breast cancer. *N Engl J Med* 347:1566–1575
- Harwell RM, Porter DC, Danes C, Keyomarsi K (2000) Processing of cyclin E differs between normal and tumor breast cells. *Cancer Res* 60:481–489
- Porter DC, Zhang N, Danes C, McGahren MJ, Harwell RM, Faruki S, Keyomarsi K (2001) Tumor-specific proteolytic processing of cyclin E generates hyperactive lower-molecular-weight forms. *Mol Cell Biol* 21:6254–6269
- Porter DC, Keyomarsi K (2000) Novel splice variants of cyclin E with altered substrate specificity. *Nucleic Acids Res* 28:E101
- Akli S, Zheng PJ, Multani AS, Wingate HF, Pathak S, Zhang N, Tucker SL, Chang S, Keyomarsi K (2004) Tumor-specific low molecular weight forms of cyclin E induce genomic instability and resistance to p21, p27, and antiestrogens in breast cancer. *Cancer Res* 64:3198–320815
- Wingate H, Puskas A, Duong M, Bui T, Richardson D, Liu Y, Tucker SL, Van Pelt C, Meijer L, Hunt K, Keyomarsi K (2009) Low molecular weight cyclin E is specific in breast cancer and is associated with mechanisms of tumor progression. *Cell Cycle* 8:1062–1068
- Akli S, Van Pelt CS, Bui T, Multani AS, Chang S, Johnson D, Tucker S, Keyomarsi K (2007) Overexpression of the low molecular weight cyclin E in transgenic mice induces metastatic mammary carcinomas through the disruption of the ARF-p53 pathway. *Cancer Res* 67:7212–7222
- Cyclacel (2007) Investigator’s Brochure Seliciclib (CYC202, R-Roscovitine) 1–88
- Smith P, Yue E (2006) CDK inhibitors of cyclin-dependent kinases as anti-tumor agents. Monographs on enzyme inhibitors. CRC Press Taylor, Francis, Boca Raton, FL
- Shapiro GI (2006) Cyclin-dependent kinase pathways as targets for cancer treatment. *J Clin Oncol* 24:1770–178320
- Malumbres M, Pevarello P, Barbacid M, Bischoff JR (2008) CDK inhibitors in cancer therapy: what is next? *Trends Pharmacol Sci* 29:16–21
- Galons H, Oumata N, Meijer L (2010) Cyclin-dependent kinase inhibitors: a survey of recent patent literature. *Expert Opin Ther Pat* 20:377–404
- Keyomarsi K, Conte D Jr, Toyofuku W, Fox MP (1995) Deregulation of cyclin E in breast cancer. *Oncogene* 11:941–950
- Pathak S (1976) Chromosome banding techniques. *J Reprod Med* 17:25–28
- Delk NA, Hunt KK, Keyomarsi K (2009) Altered subcellular localization of tumor-specific cyclin E isoforms affects cyclin-dependent kinase 2 complex formation and proteasomal regulation. *Cancer Res* 69:2817–2825
- Meijer L, Raymond E (2003) Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. *Acc Chem Res* 36:417–425
- Aldoss IT, Tashi T, Ganti AK (2009) Seliciclib in malignancies. *Expert Opin Investig Drugs* 18:1957–196527
- Benson C, White J, De Bono J, O’Donnell A, Raynaud F, Cruickshank C, McGrath H, Walton M, Workman P, Kaye S, Cassidy J, Gianella-Borradori A, Judson I, Twelves C (2007) A phase I trial of the selective oral cyclin-dependent kinase inhibitor seliciclib (CYC202; R-Roscovitine), administered twice daily for 7 days every 21 days. *Br J Cancer* 96:29–37
- Hsieh WS, Soo R, Peh BK, Loh T, Dong D, Soh D, Wong LS, Green S, Chiao J, Cui CY, Lai YF, Lee SC, Mow B, Soong R, Salto-Tellez M, Goh BC (2009) Pharmacodynamic effects of seliciclib, an orally administered cell cycle modulator, in undifferentiated nasopharyngeal cancer. *Clin Cancer Res* 15:1435–1442
- Bettayeb K, Tirado OM, Marionneau-Lambot S, Ferandin Y, Lozach O, Morris JC, Mateo-Lozano S, Drueckes P, Schachtel

- C, Kubbutat MH, Liger F, Marquet B, Joseph B, Echalié A, Endicott JA, Notario V, Meijer L (2007) Meriolins, a new class of cell death inducing kinase inhibitors with enhanced selectivity for cyclin-dependent kinases. *Cancer Res* 67:8325–8334
30. Echalié A, Bettayeb K, Ferandin Y, Lozach O, Clément M, Valette A, Liger F, Marquet B, Morris JC, Endicott JA, Joseph B, Meijer L (2008) Meriolins (3-(pyrimidin-4-yl)-7-azaindoles): synthesis, kinase inhibitory activity, cellular effects, and structure of a CDK2/cyclin A/meriolin complex. *J Med Chem* 51:737–751
31. Mettèy Y, Gompel M, Thomas V, Garnier M, Leost M, Ceballos-Picot I, Noble M, Endicott J, Vierfond JM, Meijer L (2003) Aloisines, a new family of CDK/GSK-3 inhibitors. SAR study, crystal structure in complex with CDK2, enzyme selectivity, and cellular effects. *J Med Chem* 46:222–236
32. Bagheri-Yarmand R, Biernacka A, Hunt KK, Keyomarsi K (2010) Low molecular weight cyclin E overexpression shortens mitosis, leading to chromosome missegregation and centrosome amplification. *Cancer Res* 70:5074–5084
33. Chen YN, Sharma SK, Ramsey TM, Jiang L, Martin MS, Baker K, Adams PD, Bair KW, Kaelin WG Jr (1999) Selective killing of transformed cells by cyclin/cyclin-dependent kinase 2 antagonists. *Proc Natl Acad Sci USA* 96:4325–4329
34. Ganem NJ, Godinho SA, Pellman D (2009) A mechanism linking extra centrosomes to chromosomal instability. *Nature* 460:278–282
35. Galimberti F, Thompson SL, Liu X, Li H, Memoli V, Green SR, DiRenzo J, Greninger P, Sharma SV, Settleman J, Compton DA, Dmitrovsky E (2010) Targeting the cyclin E-Cdk-2 complex represses lung cancer growth by triggering anaphase catastrophe. *Clin Cancer Res* 16:109–120
36. Lambert LA, Qiao N, Hunt KK, Lambert DH, Mills GB, Meijer L, Keyomarsi K (2008) Autophagy: a novel mechanism of synergistic cytotoxicity between doxorubicin and roscovitine in a sarcoma model. *Cancer Res* 68:7966–7974