miR Profiling Identifies Cyclin-Dependent Kinase 6 Downregulation as a Potential Mechanism of Acquired Cisplatin Resistance in Non—Small-Cell Lung Carcinoma

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Abstract

To identify the mechanisms of cisplatin resistance, global microRNA (miR) expression was tested. The expression of miR-145 was consistently higher in resistant cells. The expression of cyclin-dependent kinase 6 (CDK6), a potential target of miR-145, was lower in resistant cells, and inhibition of CDK4/6 protected cells from cisplatin. Cell cycle inhibition, currently being tested in clinical trials, might be antagonistic to cisplatin and other cytotoxic drugs.

Background: Non—small-cell lung cancer (NSCLC) is the leading cause of cancer-related death. Platinum-based chemotherapeutic drugs are the most active agents in treating advanced disease. Resistance to these drugs is common and multifactorial; insight into the molecular mechanisms involved will likely enhance efficacy. Materials and Methods: A set of NSCLC platinum-resistant sublines was created from the Calu6 cell line. Cell viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Differentially expressed microRNAs (miRs) in these lines were identified using Affymetrix miR arrays. The potential genes targeted by these miRs were searched using the TargetScan algorithm. The expression levels of miRs and mRNA were tested using real-time polymerase chain reaction. Results: miR-145 was reproducibly elevated in all the resistant sublines tested; however, modulation of miR-145 levels alone in these cells did not affect their response to cisplatin. A potential target of miR-145 is cyclin-dependent kinase 6 (CDK6), an important regulator of cell proliferation. The mRNA and protein levels of CDK6 were both downregulated in the resistant sublines. An inhibitor of CDK4/6 (PD0332991) protected parental NSCLC cells from cisplatin cytotoxicity. Conclusion: In the present study, we identified miRs differentially expressed in cisplatin-resistant cell lines, including miR-145. A predicted target of miR-145 is CDK6, and its expression was found to be downregulated in the resistant sublines, although not directly by miR-145. Inhibition of CDK6 antagonizes cisplatin-induced NSCLC cell cytotoxicity, suggesting that agents that inhibit CDK6 should be avoided during cisplatin therapy.
miR Profiling and Acquired Cisplatin Resistance in NSCLC

head and neck, cervical, and bladder cancer, with platins representing the most commonly used class of chemotherapeutic agents.2,3 In advanced NSCLC, cisplatin and carboplatin were found to have modest activity as single agents but improved the survival rates when administered in combination with other chemotherapeutic agents.4,5 Platinum-based doublets are associated with a reduced risk of death from recurrence of resected disease.6,7 Rational approaches to enhance the activity of platins in NSCLC could have significant clinical benefit; understanding the mechanisms of resistance to platins could uncover such novel therapeutic strategies.

Despite being the most efficacious chemotherapeutic agents in the treatment of NSCLC, the effectiveness of platins is limited by acquired or intrinsic resistance to these agents.8 The cellular mechanisms of resistance to platinum-based chemotherapeutic agents are multifactorial and include molecular events inhibiting drug–DNA interactions, such as a reduction in platinum accumulation inside cancer cells or inactivation by thiol-containing species.9,10 Downstream of the initial DNA damaging effects of platins, an increase in DNA adduct repair and enhanced survival signaling are additional mechanisms of platinum resistance.11,12 Increased expression of anti-apoptotic genes and mutations in the DNA-damage sensing and intrinsic apoptotic pathway could contribute to the inability of cells to detect DNA damage or to induce apoptosis.9,10 Several mechanisms of resistance could be activated in the same tumor cell by abnormal gene expression modulation.

MicroRNAs (miRs) are noncoding, short RNA molecules, each predicted to regulate hundreds of genes, apparently targeting 30% to 90% of human genes.11,12 The abnormalities of cancer cells are in part secondary to deregulation of specific miRs. Accordingly, miRs (eg, hsa-let-7, hsa-miR-155) were found to be prognostic in NSCLC13-15 and to correlate with chemotherapy resistance.16 Only ~22 nucleotides long, miRs are much more stable than the longer coding mRNAs.13,14 Identifying miRs that correlate with, and possibly regulate, platinum resistance could potentially uncover useful predictive biomarkers and might point to novel therapeutic strategies. Several algorithms have been developed to predict the genes targeted by specific miRs,17,18 although these have not been uniformly successful.19 This problem limits the insight into miR-regulated mechanisms contributing to cancer progression and therapy resistance. We report a cell line model for platinum resistance in NSCLC we have used with the aim of discovering biomarkers for platinum resistance. Through miR expression profiling, we identified cyclin-dependent kinase 6 (CDK6) downregulation to correlate with platinum resistance. Through in vitro experiments and studies of human tumor samples, we provide support for CDK6 downregulation as a potential mechanism of platinum resistance in NSCLC.

Materials and Methods

Cell Lines and Reagents

The human NSCLC tumor-derived cell lines Calu6 (HTB-56) and NCI-H23 were obtained from the American Type Culture Collection (Rockville, MD). Calu6 cells were maintained in Dulbecco’s modified Eagle’s medium and NCI-H23 cells in Roswell Park Memorial Institute medium (Media Services, Ottawa Regional Cancer Centre) supplemented with 10% fetal bovine serum (Medicorp, Montreal, QC, Canada) and glutamine (Sigma-Aldrich, St. Louis, MO).

A commercially available CDK4/6 inhibitor (CKDI) (PD0332991) was bought from Sigma-Aldrich. The miRIDIAN miR mimic was purchased from Dharmaco (Thermo Fisher Scientific, Lafayette, CO). The miR inhibitors included the miR Hairpin inhibitor from Dharmaco or anti-miR reagents from Ambion Applied Biosystems (Life Technologies, Austin, TX). PremiR-145 expression plasmid and its mutated control were a generous gift from Dr. Yin Mo.20

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

In 96-well, flat-bottom plates (Costar, New York, NY) 5000 cells/150 μL of cell suspension were seeded in each well. The cells were incubated overnight, treated, and assayed for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) activity. For analysis, 50 μL of a 5-μg/ml solution in phosphate-buffered saline of the MTT tetrazolium substrate (Sigma-Aldrich) was added and incubated for 6 hours at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 100 μL of a 0.01 M HCl/10% sodium dodecyl sulfate (SDS) solution (Sigma-Aldrich) and shaking overnight at 37°C. The plates were then read on an MRX Microplate Reader (Dynex Technologies, West Sussex, UK) at 570 nm.

Human Tumor Samples

Patients with NSCLC were retrospectively identified as having tumor resistance, defined as a ≥ 20% increase in the sum of the tumor lesion diameters during platinum-based treatment, or tumor sensitivity, defined as a reduction in tumor lesion diameters of ≥ 30%.21 Patients were identified by a manual review of the medical records focusing on those with NSCLC who had undergone previous surgical tumor resection, later developed tumor recurrence, and were treated with a platinum-based regimen at the Ottawa Hospital. The primary tumors resected at surgery, before disease progression to an advanced tumor stage, were used for molecular analysis. Tumors exposed to systemic adjuvant chemotherapy before disease recurrence were excluded. Formalin-fixed, paraffin-embedded (FFPE) tumor blocks were sectioned at 7-μm thickness. The sections were micro-dissected using laser-capture-microdissection (Arcturus, Mississauga, ON, Canada), collecting only cancer cells identified by morphology. The collected material was digested by proteinase K and frozen at −70°C until RNA extraction using the AllPrep RNA/DNA FFPE Isolation Kit (Qiagen, Valencia, CA) performed according to the manufacturer’s protocol. The average yield of RNA was approximately 230 ng/slice.

Western Blot Analysis

The cell pellets were lysed in buffer that contained 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and a protease inhibitor cocktail (all from Sigma-Aldrich). Equivalent amounts of protein were subjected to electrophoresis on SDS-polyacrylamide gel electrophoresis gels. The proteins were transferred to nitrocellulose membranes (Millipore,
Billerica, MA), and the membranes were probed with anti-CDK6 antibody (Proteintech Group, Chicago, IL), used at dilution of 1:1000 and an anti-actin antibody (Sigma-Aldrich), used at dilution of 1:5000. The immunoblots were developed using enhanced chemiluminescence (Thermo Fisher Scientific), and the images were acquired using the GeneGenome Imaging System (Syngene Bio-Imaging, Frederick, MD).

miR and mRNA Analyses
Total RNA was isolated from the cell lines in triplicate using the miRNeasy kit (Qiagen) according to the manufacturer’s instructions. miR samples for the miR microarrays were prepared with the FlashTag Biotin HSR kit (Genisphere, Hatfield, PA). miR array analysis was performed using the Affymetrix GENECHIP miRNA array according to the manufacturer’s instructions in a dedicated genomics facility at the Ottawa Hospital Research Institute (StemCore). The microarray data were analyzed using the FlexArray software package (available at: www.genomequebec.mcgill.ca/FlexArray). Quantitative polymerase chain reaction (PCR) was performed using the Real Time PCR 7500 instrument (Applied Biosystems), using TaqMan miR or TaqMan Assay kits for miR or mRNA expression (Applied Biosystems). miR reverse transcription (RT) quantitative (Q)-PCR results were normalized with miR-191 expression, and mRNA Q-PCR results were normalized with glyceraldehyde-3-phosphate dehydrogenase expression.

Statistical Analysis
The growth rate of the cells, quantified protein blots, and RT-PCR results were compared using Student’s *t* test. MiR arrays were analyzed using the LIMMA package algorithm. Modulated *t* tests were performed, and results were corrected for multiple comparisons (false discovery rate method), with a *q* value < .05 considered significant. Student *t* tests were performed to assess the statistical significance of the CDK6 expression levels in the NSCLC tumors evaluated. Similar analyses were performed to evaluate the significance of the combination therapies, in which each treatment alone was compared with the combination, and significance was attributed when both agents used alone were significant compared with the combination treatments.

Ethics
The Ottawa Hospital Research Ethics Board approved the medical record review for the identification of platinum-sensitive and -resistant cases and the molecular analysis of the tumors.

Results
Identification of miRs Differentially Expressed in NSCLC-Calu6 Cisplatin-Resistant Sublines
A series of cisplatin-resistant clones were developed from the Calu6 cell line. Calu6 originates from an anaplastic NSCLC, known to harbor genetic mutations in KRAS and TP53, commonly found in lung cancer. To derive the cisplatin-resistant sublines, parental cells were plated and treated with 2 μg/mL cisplatin until a surviving fraction of about $10^{-6}$ remained. The surviving colonies were isolated and propagated for > 20 population doublings. Calu6 cisplatin-resistant sublines were designated E37, numbered 1 through 12. The inhibitory constant at 50% cell viability (IC50) after 48 hours of cisplatin treatment was subsequently determined using an MTT assay (Figure 1A). As expected of the diversity of cancer cells surviving chemotherapy, each resistant subline demonstrated a different level of resistance, with the IC50 varying within 1.75 to 7.35 times the IC50 of the parental line. The Calu6 cisplatin-resistant sublines demonstrated a similar but
MicroRNA (miR) Profiling of Calu6 and Cisplatin-Resistant Sublines Identified Differentially Expressed miRs. (A) Study Scheme. Three Replicates of the Sensitive Parental Calu6 Were Compared With 3 Replicates of Each of 3 Cisplatin-Resistant Sublines. (B) All miRs With Fold Differences > 2, Shown With Average Expression of Sensitive and Resistant Lines: Fold Difference (Resistant vs. Sensitive), \( P \) Value, and Q Value (\( P \) Values Corrected for Multiple Comparisons). (C) Average Expression of miR145 in Sensitive (\( n = 3 \)) and Resistant (\( n = 9 \)) Cell Lines as Measured in miR Arrays. Error Bars Demonstrate SD.
After transfection of the mimic (Figure 3B) or pre-miR-145 expression construct (data not shown), the Q-PCR MTT cell viability assay (Figure 3C). In addition, the use of an miR-145 inhibitor (hairpin inhibitor, Dharmacon; and anti-miR, Ambion) on the platinum-resistant sublines did not modulate

The goal of establishing these resistant sublines was to identify differentially expressed miRs that might play a role in the cisplatin-resistant phenotype. Three independent RNA extractions and microarray analyses were performed from the parental sensitive lines and from each of the most resistant sublines. We used the Affymetrix miRNA microarrays, which can assay 847 known mature human miRs, encompassing approximately 90% of the recognized miRs at analysis, which are 32% of the 2578 mature miRs currently registered in miRBase. The parental Calu6 (triplicate) was compared with the resistant sublines (3 replicates of each resistant line, total of 9; Figure 2A). Eight miRs were found to have a fold difference of expression > 2 between the resistant and sensitive lines (Figure 2B). The highest fold difference was seen for miR-145, with a 4.6-fold higher expression in the Calu6 resistant sublines than in the parental Calu6 line (Q value = 0.01; Figure 2C). The next miR in this regard was miR-205, which was 3.8-fold higher in the resistant lines. For miR-205 and the rest of the miRs with a > 2-fold difference in expression, the differences were not statistically significant. Two additional miRs had a difference of expression that was statistically significant; however, owing to the low difference in expression, they were not studied further (hsa-miR-425 at 1.09-fold and hsa-miR-378 at 0.9-fold; data not shown). Because miR-145 has previously been demonstrated to play a role in mediating cisplatin resistance in a variety of tumor cell lines models,24,25 we chose to evaluate this miR in particular on the cisplatin response.

The expression differences of miR-145 seen in the miR array results of the Calu6 lines were validated using Q-PCR on a larger set of the resistant Calu6 cell lines. Six E37 sublines were chosen, encompassing a range of cisplatin IC50 values. All the resistant sublines demonstrated enhanced miR-145 expression in the range of 8- to 35-fold compared with the parental Calu6 line, confirming that overexpression of miR-145 is a common feature of the E37 resistant sublines (Figure 3A). The range of miR-145 expression between the sublines highlights that each subline was the outgrowth of a different platinum-surviving cell, with the expected variability in the properties of each cell.

To determine the potential of miR-145 to modulate cisplatin sensitivity in the Calu6 cell line, we transfected a miR mimic of miR-145, expected to have biologic activity similar to that of a mature miR, or a pre-miR-145 construct,20 and assayed for miR-145 expression and sensitivity to cisplatin-induced cytotoxicity. After transfection of the mimic (Figure 3B) or pre-miR-145 expression construct (data not shown), the Q-PCR–detected miR-145 levels were induced to almost 1000 times the Calu6 endogenous levels, which were well above the levels determined in the Calu6 or in the E37 resistant clones. However, cells highly expressing exogenous miR-145 showed no significant effect with respect to cisplatin-induced cytotoxicity, determined using the MTT cell viability assay (Figure 3C). In addition, the use of an miR-145 inhibitor (hairpin inhibitor, Dharmacon; and anti-miR, Ambion) on the platinum-resistant sublines did not modulate

Figure 3 MicroRNA-145 (miR-145) Expression and Sensitivity to Cisplatin. A, Relative Expression of miR-145 as Determined by Quantitative Polymerase Chain Reaction (Q-PCR) in the Calu6 (Normalized to 1.0 for Ease of Presentation) and 6 of Its Cisplatin-Resistant E37 Sublines. All miR-145 Levels in the Sublines Differed From Those in Calu6 (P < .001; t Test), miR-145 Levels Were Corrected to miR-191 Levels. (B) Calu6 Cells Were Transfected With a miR-145 Mimic (40 nM, miRIDIAN) Using Altogen Calu6 Transfection Reagent. Green Fluorescent Protein Expression Plasmid and an Inert Fluorescent Oligonucleotide Were Cotransfected and Demonstrated a > 80% Transfection Efficiency. Controls Included a Nontreated Culture, Mock Transfection, and Cells Transfected With a miR Mimic Control. Three Days After Transfection, Total RNA Was Extracted, and miR-specific Q-PCR Was Performed to Determine the Levels of miR-145 and miR-191. (C) In Parallel With the Experiment Described in (B), Cells Similarly Transfected in 96-well Plates Were Treated 24 Hours Later With Cisplatin at the Indicated Concentrations. After 48 Hours of Treatment, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay Was Performed to Evaluate Effects of Treatment on Cell Viability. All Error Bars Indicate SD

Abbreviation: RQ = Relative quantitation.
Cisplatin sensitivity (data not shown). These results suggest that cisplatin resistance in the Calu6 resistant sublines is not directly mediated by miR-145.

**CDK6 as a Potential miR-145 Target and Cisplatin Sensitivity Mediator**

Following our observation of a slightly slower growth of the Calu6 resistant sublines (Figure 1B), we speculated that a cell cycle driving gene might be a target of miR-145 and that its reduction could regulate the cisplatin-resistance phenotype. Using the TargetScanHuman online algorithm (release 6.2), we interrogated this possibility regarding the main genes known to be involved in cell cycle progression. We identified in the 3'UTR of one of these genes, CDK6, one 8-mer and one 7-mer conserved sites that fit the seed sequence of miR-145. The corresponding probability of genes, CDK6, one 8-mer and one 7-mer conserved sites that fit the seed sequence of miR-145. The corresponding probability of genes, CDK6 as a putative mediator of cisplatin sensitivity.

We evaluated the CDK6 protein levels in parental Calu6 cells and E37-1, E37-10, and E37-11, the Calu6 sublines with the highest IC_{50} for cisplatin (Figure 1A), and E37-4, a less-resistant subline but with the greatest expression of miR145 (Figure 3A). In all cases, the E37 sublines expressed reduced levels of CDK6, which were unaffected by cisplatin treatment (Figure 4A). Quantiﬁcation of the Western blot shown in Figure 4B demonstrated the CDK6 protein levels (corrected for actin levels) of E37-1 and of E37-11 to differ signiﬁcantly (P < .05) compared with those of Calu6 (data not shown). The CDK6 mRNA levels were also reduced in the resistant lines; however, the reduction was not as large as that seen for the protein (Figure 4C). Therefore, the Calu6 platinum-resistant sublines that expressed high levels of miR-145 compared with the parental sensitive line, also expressed low levels of CDK6 compared with the parental line.

Aiming to substantiate the correlation of low CDK6 levels with cisplatin resistance, we retrospectively identiﬁed several cases of clinical cisplatin-resistant and -sensitive NSCLC. RNA extracted by microdissection of the cancer cells from these samples was evaluated for the CDK6 mRNA levels. The CDK6 mRNA levels were signiﬁcantly lower in the resistant cases (n = 3) than in the platinum-sensitive cases (n = 2; Figure 4D).

Next, we evaluated the potential of miR-145 and miR-205, the miRs found to differ maximally between the parental Calu6 and their cisplatin-resistant sublines, to reduce the CDK6 expression levels in the Calu6 parental line. The TargetScan algorithm identiﬁed miR-145 target sites in the 3'UTR of CDK6, as mentioned, but no miR-205 target sequence was identiﬁed in the CDK6 gene. To examine experimentally the prediction of CDK6 downregulation by miR-145, the miR-145 levels were enhanced by transfection of the miR-145 mimic or inhibited by speciﬁc inhibitors (anti-miR-145 hairpin inhibitors from Dharmacon and Ambion). However, neither could modulate CDK6 expression (Figure 4E). Because, miR-205 could potentially target CDK6 in an indirect manner, we combined both anti-miR-145 and anti-miR-205 and did not ﬁnd any changes in CDK6 expression in the Calu6 cells (Figure 4E). Thus, we could not demonstrate that these speciﬁc miRs control the levels of CDK6 in Calu6 cells.

**Inhibition of CDK4/6 Antagonizes Cisplatin-Induced Cytotoxicity**

If the lower levels of CDK6 found in the cisplatin-resistant E37 sublines play a role in the mechanism of resistance, inhibition of CDK6 should protect cells from cisplatin cytotoxicity. To test this assumption, a commercially available CDK4/6 inhibitor (PD0332991) was used. MTT assays were performed in Calu6 (the parental, platinum-sensitive line) and E37-10 cells (the most resistant subline in our set) treated with cisplatin alone or combined with 10-μM CDKI for 48 hours. The 10-μM dose of the CDKI alone reduced MTI activity by 50% in both lines. The combination of the CDKI and cisplatin failed to demonstrate enhanced cytotoxic activity compared with either agent alone in both lines and appeared to be antagonistic (Figure 5). Using an alternative method and additional cell lines, trypan blue exclusion was determined for Calu6 and NCI-H23 cells. The addition of CDKI treatment induced loss of cell viability on its own, failed to enhance cisplatin-induced cytotoxicity in either cell line, and, in some dose combinations, reduced cisplatin-induced cytotoxicity, further demonstrating that cisplatin and CDKI were antagonistic in our experimental system (Figure 5B).

**Discussion**

Using an NSCLC cell-line model of acquired cisplatin resistance we generated from the parental Calu6 cell line, we found CDK6 downregulation to correlate with resistance and CDK4/6 inhibition to be antagonistic to cisplatin. Our results resonate with the classic notion that rapid cell cycle progression is the phenotype that dictates the chemo- and radiosensitivity of cancer cells compared with normal cells. However, our ﬁndings are in contrast to reports of cisplatin sensitivity being maximal in the G1 phase. Inhibiting G1 progression, an expected outcome of CDK6 downregulation, would be predicted to enhance susceptibility to cisplatin, not reduce it, which we have demonstrated. The CDK4/6 inhibition used in our experiments inhibited cell proliferation of lung cancer cells (Figure 5). Because CDK4/6 inhibition has been considered as a potential anticancer treatment, it is important to note that our results suggest caution in its use when combined with platinum agents.

Elevated expression of miR-145 was strongly associated with cisplatin resistance in a large set of independently generated Calu6 sublines. Although we could not demonstrate a role for miR-145 in mediating cisplatin resistance, this miR merits additional investigation. Cisplatin resistance is driven by a wide range of pleiotropic cellular mechanisms, and miR-145 might play a role without manifesting the resistance phenotype when modulated in isolation. The stability of miRs in various biologic specimens, including tumor tissue, supports their use as predictive and prognostic biomarkers. The ongoing elevated expression of miR-145 in each of the E37 resistant cell lines after > 20 passages without exposure to
cisplatin implicates the potential importance and a need for additional exploration of its role in platinum resistance. The identification of miR-145 and CDK6 as potential predictors of platinum responsiveness could allow for personalized treatments regarding cisplatin and carboplatin, improving the efficacy and reducing the unnecessary toxicity of these powerful cytotoxic agents. Validation of our results in a large set of human NSCLC specimens is required to suggest its use clinically.

Failure to demonstrate the targeting of a specific gene by a single miR might indicate that this specific miR requires other coregulators to effectively modulate the expression of this gene. For example, a number of miRs, in combination and additional signaling pathways, might cooperatively regulate the expression of a common gene (eg, CDK6), leading to an observed phenotypic change (ie, cisplatin resistance). The complexity of miR-mRNA targeting networks and possible off-target effects of exogenous miR-mimics or miR-antagonists potentially hinders studies evaluating the role of a single miR, such as our study. Regardless, our identification of CDK6 as a potential mediator of cisplatin sensitivity requires additional evaluation.
Conclusion
Through a miR-based screen, we identified the expression of miR-145 to correlate with cisplatin resistance in NSCLC cells. This result led us to examine CDK6 as a mediator of cisplatin sensitivity and to demonstrate that CDK4/6 inhibition antagonizes cisplatin cytotoxicity. This finding should be considered when clinically assessing such combinations.

Clinical Practice Points
- Cisplatin and related agents are among the most active drugs against lung cancer.
- Resistance to these drugs invariably evolves, usually followed by rapid tumor progression.
- Targeted agents are a promising modality, currently being tested in clinical trials, either alone or concomitantly with chemotherapy agents.
- Among these are inhibitors of cell cycle progression such as CDK inhibitors.
- Using cisplatin-resistant lung cancer cells, we tested global miR expression, identifying miR-145 to correlate with cisplatin resistance.
- We found CDK6 to associate with cisplatin sensitivity, and inhibition of CDK4/6 to be antagonistic to cisplatin cytotoxicity.
- This finding has important implications for future clinical trial designs.
- CDKIs and other cell cycle inhibitors should be used cautiously in combination with cisplatin and similar cytotoxic drugs since combining cisplatin and similar cytotoxic agents with CDK4/6 inhibitors or other cell cycle inhibitors could be antagonistic.

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Disclosure

The authors have stated that they have no conflicts of interest.

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